INTERACTION OF HAEMOGLOBIN, NITRIC OXIDE AND THE STRESS PROTEIN HAEM OXYGENASE-1: FUNCTIONAL IMPLICATIONS IN SICKLE CELL DISEASE

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THESIS SUBMITTED FOR DOCTOR OF PHILOSOPHY IN THE UNIVERSITY OF LONDON

2007

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Abstract

Haemoglobin and nitric oxide (NO) strongly induce the stress protein haem oxygenase-1 (HO-1), which degrades haem to carbon monoxide (CO), a signalling and vasoactive molecule, the anti-oxidant biliverdin (BV) and iron. Raised HO-1 levels have been detected in the endothelium and kidneys of animals, as well as leukocytes of patients, suffering from sickle cell disease (SCD). A genetic mutation of haemoglobin causes SCD, pathologic symptoms of which include vaso-occlusive crises and high concentrations of free haemoglobin liberated during red blood cell haemolysis. Although HO-1 and its products have been linked to SCD, their potential role in this condition has not been examined. The work of this thesis aimed to: 1) investigate the interaction of haemoglobin and NO in modulating endothelial HO-1 expression; 2) examine the effect of human sickle cell blood on HO-1 induction and vascular function; and 3) assess the role of HO-1 and its products on sickle cell blood adhesion and regulation of vessel contractility. Experiments were performed using a combination of *in vitro* (cell culture and biochemical assays) and *ex-vivo* (isolated aortic rings) models. The results of the study indicated that NO synergises with haemoglobin to amplify HO-1 expression and haem incorporation by endothelial cells, suggesting that similar mechanisms might contribute to changes in vascular function occurring in haemolytic disorders. It was also found that sickle cell blood induces haem oxygenase to a greater extent than normal blood, an effect which is magnified under hypoxia. The increased haem oxygenase elicited by sickle cell blood depends on the time elapsed since the last vaso-occlusive crisis experienced by the patient. Finally, CO and BV diminish sickle blood adhesion to the endothelium and sickle blood

can alter CO-mediated vessel relaxation. These findings support a functional role for the HO-1 pathway in SCD and may help to identify therapeutic strategies to counteract the vascular damage caused by SCD.

Acknowledgements

All research undertaken in this Thesis was carried out by Sandip Bains, but would not have been possible without the continual support of Dr Roberto Motterlini, my supervisor and Head of the Vascular Biology Unit, Professor Colin Green, Director of the Northwick Park Institute of Medical Research and Dr Roberta Foresti for their guidance and encouragement throughout the study.

I would also like to thank the many current and former members of the Department of Surgical Research at the Northwick Park Institute of Medical Research who have been invaluable with their support whilst working on this project.

List of Contents

Abstract ..... **i**

Acknowledgements ..... **iii**

List of Contents ..... **iv**

List of Figures ..... **xiii**

List of Abbreviations ..... **xix**

List of Abbreviations ..... **xix**

Chapter 1. Introduction **1**

1.1 The haem oxygenase pathway **1**

1.1.1 History of haem oxygenase 1

1.1.2 Carbon monoxide 4

1.1.3 Biliverdin and Bilirubin 6

1.1.4 Iron 7

1.1.5 Induction and regulation of HO-1 8

1.1.6 HO-1 and signal transduction 12

1.1.7 HO-1 as a therapeutic target 15

1.1.8 CO-releasing molecules 17

1.2 Haem **21**

1.2.1 Haem synthesis 22

1.2.2 Haem-binding proteins 24

1.2.3 Free haem detoxification systems 26

1.2.4 Haem receptors 28

1.2.5 Haem in the circulation: effects on the vascular endothelium 29

1.2.6 Haem and oxidative stress 32

1.2.7 Haem and LDL oxidation 33

1.2.8 Haem and inflammation 34

1.3 Haemoglobins 35

1.3.1 Chemical structure and properties 35

1.3.2 Physiological role of haemoglobins 37

1.3.3 Haemoglobin gene regulation 38

1.3.4 Haemoglobinopathies 39

1.4 Sickle cell disease (SCD) 40

1.4.1 Pathophysiology of sickle cell disease 42

1.4.2 Treatment of sickle cell disease 44

1.4.3 Haem oxygenase and sickle cell disease 46

1.5 Hypoxia 50

1.5.1 Hypoxia inducible factor-1 51

1.5.2 Erythropoietin 51

1.5.3 Hypoxia and haem oxygenase 52

1.5.4 Hypoxia and sickle cell disease 53

1.6 Nitric oxide 55

1.6.1 Chemical properties and reactions of NO 55

1.6.2 NO synthesis 56

1.6.3 Interaction of NO with biological molecules 58

1.6.4 NO and haemoglobin binding 59
1.6.5 NO and haemoglobin interaction: physiological implications 60
1.6.6 NO and haemoglobin interaction: pathophysiological implications 62
1.6.7 Haem-nitrosyl complex formation 63
1.6.8 NO interaction with haem oxygenase 63
1.6.9 Up-regulation of HO-1 by NO 64
1.6.10 Modulation of haem oxygenase activity by NO 66
1.6.11 Interaction of NO with haem metabolites 67
1.6.12 Potential effect of haem and NO interaction on HO-1 68

1.7 Hypothesis and objectives 69

Chapter 2. Materials and Methods 70

2.1 Reagents 70

2.2 Synthesis of ferrous haem-nitrosyl (H-NO) 70

2.3 Synthesis of CO-releasing molecules (CO-RMs) 72
2.3.1 Synthesis of tricarbonylchloro(glycinato)ruthenium (II) (CORM-3) 72
2.3.2 Synthesis of sodium boranocarbonate (CORM-A1) 72
2.3.3 Release of CO from CORM-3 and CORM-A1 73

2.4 Cell culture 75
2.4.1 Bovine aortic endothelial cells 76
2.4.2 Porcine renal epithelial cells (LLC-PK_1) 77
2.4.3 Human adult atrial myoblast cells (Girardi) 77
2.4.4 RAW 264.7 murine macrophages 77

2.4.5 Human erythroid progenitor cells (K562) 77

2.4.6 Subculture of cells for incubation 78

2.5 Collection of samples 78

2.6 Preparation of liver cytosol and liver microsomes 79

2.7 Assay for endothelial haem oxygenase activity 80

2.8 Protein determination 81

2.9 Western-blot technique for detection of HO-1 protein expression 82

2.10 Assay for determination of cellular haem content 86

2.11 Determination of bilirubin in culture medium 87

2.12 Nitric oxide electrode 87

2.13 Endothelial cell adhesion assay 89

2.14 Isolated rat aortic ring 91

2.15 Lactate dehydrogenase assay 92

2.16 Statistics 94

Chapter 3. Effect of NO and haemoglobin interaction on endothelial haem oxygenase induction 95

3.1 Introduction 95

3.2 Objectives 96

3.3 Experimental protocol **97**

3.3.1 Incubation of cells with reagents 97

3.3.2 Haem oxygenase activity assay 98

3.3.3 Western-blot techniques for detection of HO-1 protein expression 99

3.3.4 Determination of bilirubin in culture medium 99

3.3.5 NO release measurement 100

3.4 Results **101**

3.4.1 Both NO^+ (GSNO) and NO^- (AS) donors synergize with haemoglobin to increase endothelial haem oxygenase activity and up-regulate HO-1 expression 101

3.4.2 Comparison between haem-nitrosyl and haemin on the stimulation of haem oxygenase activity and bilirubin production 108

3.4.3 Co-incubation of hydroxyurea with haemin or haemoglobins enhances endothelial haem oxygenase activity 110

3.4.4 NO release from AS and GSNO in medium is affected by the presence of haemoglobins 113

3.4.5 NO release from hydroxyurea in the presence or absence of haemoglobins 116

3.5 Discussion **118**

Chapter 4. Effect of NO and haemoglobin interaction on endothelial haem uptake **124**

4.1 Introduction **124**

4.2 Objectives **126**

4.3 Experimental protocol 127

4.3.1 Incubation of cells with reagents..... 127

4.3.2 Assay for determination of cellular haem content..... 128

4.4 Results 129

4.4.1 Effect of AS on endothelial haem uptake elicited by haemoglobins..... 129

4.4.2 Comparison between haem-nitrosyl and haemin on changes of cellular haem levels..... 132

4.4.3 Effect of bovine albumin on increased haem uptake mediated by AS. 134

4.4.4 Measurements of haem uptake in different cell types..... 137

4.4.5 Co-incubation of hydroxyurea with haemin or haemoglobin does not affect endothelial haem uptake 140

4.5 Discussion 143

Chapter 5. Effect of sickle blood on endothelial haem oxygenase..... 148

5.1 Introduction 148

5.2 Objectives 150

5.3 Experimental protocol 151

5.3.1 Patient population..... 151

5.3.2 Incubation of cells with reagents..... 152

5.3.3 Haem oxygenase activity assay 153

5.3.4 Western-blot technique for detection of HO-1 protein expression 153

5.3.5 Statistical analysis 154

5.4 Results 155

5.4.1 Experimental results 155

5.4.2 Analytical results 169

5.5 Discussion 186

Chapter 6. Haem oxygenase and its products regulate the interaction of sickle blood with the vascular endothelium 199

6.1 Introduction 199

6.2 Objectives 200

6.3 Experimental protocol 201

6.3.1 Determination of red blood cell adhesion to endothelium 201

6.3.2 Experiments using the isolated aortic ring model 203

6.3.3 Statistics 204

6.4 Results 205

6.4.1 Optimisation of the assay for quantifying the adhesiveness of sickle red blood cell to endothelial cells 205

6.4.2 Haemolysis of blood from normal subjects does not induce red blood cell adherence to endothelium 207

6.4.3 Induction of haem oxygenase and haem oxygenase-derived products modulate the adhesion of sickle red blood cells to the endothelium 208

6.4.4 Sickle whole blood modulates the vasodilatory effect of CORM-3 and CORM-A1 on isolated aortic rings 214

6.4.5 Sickle red blood cells modulate the vasodilatory effect of CORM-3 and CORM-A1 on isolated aortic rings 218

6.4.6 Sickle whole blood modulates the vasodilatory effect of NO donors on isolated aortic rings 222

6.5 Discussion 225

Chapter 7. Exploring the effect of HO-1 inducers on erythroid progenitor cells (K562) 235

7.1 Introduction 235

7.2 Objectives 237

7.3 Experimental protocol 238

7.3.1 Incubation of cells with reagents 238

7.3.2 Haem oxygenase activity assay 239

7.3.3 Lactate dehydrogenase assay 240

7.4 Results 241

7.4.1 Response of K562 cells to classic HO-1 inducers 241

7.4.2 Effect of hydroxyurea on haem oxygenase activity in K562 cells 245

7.4.3 Co-incubation of K562 cells with haem and hydroxyurea: haem oxygenase activity response and cytotoxicity experiments 249

7.4.4 Effect of haem oxygenase degradation products on the cytotoxicity induced by H_2O_2 in K562 cells 254

7.5 Discussion 256

Chapter 8. General Discussion 261

8.1 Analysis of methodology 261

8.1.1 Cell culture methodology 261

8.1.2 Biochemical and molecular biology techniques 262

8.1.3 Endothelial cell adhesion assay 265

8.1.4 Isolated aortic ring 266

8.1.5 Limitations of the study 267

8.2 Hypothesis and objectives 269

8.3 General discussion 271

8.4 Conclusion 285

8.5 Future perspectives 286

Chapter 9. List of publications 288

Chapter 10. References 289

Chapter 11. Appendix 341

11.1Appendix 1: Full publications by the author 341

List of Figures

Figure 1-1. The haem oxygenase pathway 3

Figure 1-2. Induction of HO-1 9

Figure 1-3. Haem synthesis 23

Figure 1-4. Structure of adult mammalian haemoglobin 37

Figure 1-5. Deoxygenation triggers sickling of red blood cells 41

Figure 1-6. NO synthesis and action 57

Figure 2-1. Spectrum of H-NO generation 71

Figure 2-2. Release of CO from CORM-3 and CORM-A1 in aqueous solutions 73

Figure 2-3. Rate of CO release from CORM-3 vs. CORM-A1 74

Figure 2-4. Representative standard curve for protein determination 82

Figure 2-5. Arrangement of gel sandwich assembly 84

Figure 2-6. Binding cascade for development of bands for HO-1 Western blot 86

Figure 2-7. NO electrode experimental set-up 88

Figure 2-8. Representative standard curve for NO measurement 89

Figure 2-9. Summary of red blood cell-endothelial cell adhesion assay protocol 90

Figure 2-10. Overview of isolated aortic ring experimental set-up 92

Figure 2-11. Principle behind lactate dehydrogenase colorimetric assay 93

Figure 3-1. GSNO synergizes with methaemoglobin to up-regulate endothelial haem oxygenase activity and HO-1 expression 104

Figure 3-2. GSNO synergizes with HbA_0 to up-regulate endothelial haem oxygenase activity and HO-1 expression 105

Figure 3-3. GSNO synergizes with HbS to up-regulate endothelial haem oxygenase activity and HO-1 expression 106
Figure 3-4. A nitroxyl generator synergizes with different haemoglobins to up-regulate endothelial haem oxygenase activity 107
Figure 3-5. Effect of haem-nitrosyl on haem oxygenase activity, HO-1 expression and bilirubin production 109
Figure 3-6. Effect of hydroxyurea alone or in combination with haem on haem oxygenase activity 111
Figure 3-7. Hydroxyurea synergizes with different haemoglobins to up-regulate endothelial haem oxygenase activity 112
Figure 3-8. Release of NO from GSNO in the presence of haemoglobins 114
Figure 3-9. Release of NO from AS in the presence of haemoglobins 115
Figure 3-10. Release of NO from hydroxyurea in the presence of haemoglobins 117
Figure 4-1. A nitroxyl generator synergizes with different haemoglobins to up-regulate endothelial haem uptake 131
Figure 4-2. Effect of haem-nitrosyl complex (H-NO) on haem uptake 133
Figure 4-3. Pre-incubation with AS and bovine serum albumin modulate haem uptake by endothelial cells 136
Figure 4-4. Haem uptake in different cell types 139
Figure 4-5. Effect of hydroxyurea and haemin on haem uptake 141
Figure 4-6. Effect of hydroxyurea and haemoglobins on haem uptake 142
Figure 5-1. Breakdown of sickle variants in SCD patient population 156
Figure 5-2. Endothelial haem oxygenase activity elicited by whole blood solutions 161

Figure 5-3. Sickle blood induces haem oxygenase in endothelial cells..... 162
Figure 5-4. Hypoxia amplifies the effect of sickle blood on haem oxygenase induction in endothelial cells..... 164
Figure 5-5. Hypoxia amplifies the effect co-incubation of hydroxyurea with haemoglobin on HO-1 expression in endothelial cells..... 165
Figure 5-6. Co-incubation of NO donors with sickle blood does not affect haem oxygenase induction in endothelial cells 167
Figure 5-7. Hydroxyurea does not amplify the induction of haem oxygenase mediated by sickle blood in hypoxic conditions..... 168
Figure 5-8. Patient treatment regimen modulates haem oxygenase induction171
Figure 5-9. Disease state influences endothelial haem oxygenase activity and haemoglobin levels 173
Figure 5-10. BR and LDH levels in blood of patients vary according to disease state 174
Figure 5-11. The time elapsed since the last vaso-occlusive crisis influences haem oxygenase activity levels..... 176
Figure 5-12. The time elapsed since the last vaso-occlusive crisis influences BR and LDH levels in blood 177
Figure 5-13. The frequency of sickle crises influences and endothelial haem oxygenase activity stimulated by blood..... 179
Figure 5-14. Correlation between endothelial haem oxygenase activity and haemoglobin levels 181
Figure 5-15. Correlation between haem oxygenase activity and BR levels.... 182
Figure 5-16. Correlation between endothelial haem oxygenase activity elicited by sickle blood and LDH levels 184

Figure 5-17. Correlation between endothelial haem oxygenase activity elicited by sickle blood and patient age 185
Figure 5-18. Possible mechanism of action for haem oxygenase induction after exposure of endothelium to sickle blood 195
Figure 6-1. Adherence of red blood cells to the endothelium using normal or sickle blood 206
Figure 6-2. Pre-incubation with haemin decreases the adherence of sickle red blood cells (SC-RBC) to the endothelium 210
Figure 6-3. Pre-incubation with biliverdin reduces the adherence of sickle red blood cells (SC-RBC) to the endothelium 211
Figure 6-4. Pre-treatment with CORM-3 modulates adherence of sickle red blood cells to the endothelium 212
Figure 6-5. Pre-treatment with CORM-A1 reduces adherence of sickle red blood cells (SC-RBC) to the endothelium 213
Figure 6-6. Sickle blood modulates vessel relaxation in response to CORM-A1 216
Figure 6-7. Sickle and normal blood modulates vessel relaxation in response to CORM-3 217
Figure 6-8. Sickle red blood cells modulate vessel relaxation in response to CO-RMs 220
Figure 6-9. Sickle plasma modulate vessel relaxation in response to CO-RMs 221
Figure 6-10. Sickle blood modulates vessel relaxation in response to NO donors 224

Figure 6-11. Proposed role for haem oxygenase in prevention of vaso-occlusion in SCD 233
Figure 7-1. Classic HO-1 inducers do not increase haem oxygenase activity in K562 cells 243
Figure 7-2. Haemin reduces haem oxygenase activity of K562 cells in a concentration-dependent manner 244
Figure 7-3. Hydroxyurea affects haem oxygenase activity in K562 cells: concentration and time-dependent effects 247
Figure 7-4. Thymidine affects haem oxygenase activity in K562 cells 248
Figure 7-5. Hydroxyurea synergizes with haemin to down regulate haem oxygenase activity in K562 cells 251
Figure 7-6. Haemin and hydroxyurea provide protection against oxidant-induced damage 252
Figure 7-7. A haem oxygenase inhibitor does not affect the protection elicited by haemin or hydroxyurea against oxidant-mediated damage in K562 cells 253
Figure 7-8. CO and BV protect K562 cells against oxidant-induced damage. 255
Figure 7-9. Potential role of haem oxygenase in K562 cells 260
Figure 8-1. Possible mechanisms underlying enhanced haem oxygenase induction by NO, haemoglobin and haem 274
Figure 8-2. Potential effects of hydroxyurea on haem oxygenase in SCD 277
Figure 8-3. Haem oxygenase induction and potential therapeutic action in SCD 284

List of Tables

Table 2-1. Culture medium of different cell types 76

Table 5-1. Patients characteristics 157

Table 5-2. Plasma analysis of sickle patient subset 158

Table 5-3. Patient characteristics separated by hydroxyurea treatment..... 170

Table 5-4. Patient characteristics separated by transfusion regimen 170

Table 5-5. Pearson correlations between haemoglobin levels in male or female patients and BR, LDH levels or age 183

Table 6-1. Red blood cell adherence of haemolysis in control blood. 207

List of Abbreviations

ALA	5-aminolevulinic acid
ALAD	ALA dehydratase
ALAS	δ-aminolevulvinate synthase
AP-1	Activating protein-1
AS	Angeli's salt
BAEC	Bovine aortic endothelial cells
BR	Bilirubin
BV	Biliverdin
BVR	Biliverdin reductase
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CNC-bZIP	Cap'N'Collar-basic-leucine zipper
CO	Carbon monoxide
CO-RM	Carbon monoxide releasing molecule
COREC	Central Office of Research Ethics Committees
DMEM	Dulbecco's modified eagles medium
DPG	2, 3-diphosphoglycerate
EDRF	Endothelium derived relaxing factor
EDTA	Ethylenediamine tetraacetic acid
eNOS	Endothelial NOS
ERK	Extracellular signal regulated kinase

E-selectin	endothelial leukocyte adhesion molecules
Fe	Iron
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GSH	Glutathione
GSNO	S-Nitrosoglutathione
H_2O	Water
Hb	Haemoglobin
HbA	Adult mammalian haemoglobin
HbF	Foetal haemoglobin
HbNO	Nitrosyl haemoglobin
HBP	Haem binding protein
HbS	Sickle haemoglobin
HCP1	Haem carrier protein 1
HDL-C	High-density lipoprotein cholesterol
H-NO	Haem-nitrosyl
HO	Haem oxygenase
Hp	Haptoglobin
HPFH	Hereditary persistence of foetal haemoglobin
HU	Hydroxyurea
Hx	Haemopexin
ICAM	Intracellular adhesion molecule

iNOS	Inducible NOS
JNK	c-jun N-terminal kinase
LDH	Lactate Dehydrogenase
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MEL	Murine erythroleukemia cells
MetHb	Oxidised haemoglobin
MKK	MAPK kinase
MKKK	MKK kinase
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium Hydroxide
NF-E2	Nuclear factor-erythroid 2
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
NO^-	Nitroxyl anion
NO^+	Nitrosonium cation
NO_2^-	Nitrite
NOS	Nitric oxide synthase
NrF	(NF-E2)-related factor
O_2	Oxygen
O_2^-	Superoxide
$ONOO^-$	Peroxynitrite

PBG	Porphobilinogen
PKA	Protein kinase A
PKC	Protein kinase C
PMN	Polymorphonuclear cell
PO_2	Partial pressure of oxygen
RBC	Red blood cell
ROS	Reactive oxygen species
SCD	Sickle cell disease
sGC	Soluble guanylate cyclase
SNO-Hb	S-nitroso-haemoglobin
SnPPIX	Tin protoporphyrin IX
StRE	Stress response element
TBARS	Thiol barbituric acid reactive substance
UVA	Ultra violet radiation
VCAM1	Vascular cell adhesion molecule1

Chapter 1. Introduction

1.1 The haem oxygenase pathway

1.1.1 History of haem oxygenase

The degradation of haem to biliverdin (BV) was first demonstrated *in vitro* by Wise and Drabkin (Wise CD. and Drabkin DL, 1964). The protein responsible for this degradation was subsequently demonstrated to be haem oxygenase by Tenhunen (Tenhunen et al., 1969), who confirmed that this enzyme is responsible for the catabolism of haem as well as characterizing the enzyme and its cellular location. The initial presumption was that haem oxygenase was a member of the cytochrome P450 family of enzymes (Tenhunen et al., 1969); however, subsequent investigations established haem oxygenase as a rapidly and transiently inducible mono-oxygenase that functioned independent of cytochrome P450 (Maines et al., 1986). The inducible isoform of haem oxygenase, haem oxygenase-1 (HO-1), was later found to be identical to heat shock protein 32 and this resulted in interest in HO-1 as a stress response protein. The production of specific stress proteins is the cellular response to metabolic and stressful changes and HO-1 was found to be induced by various stimuli that directly and indirectly exert oxidative stress. Therefore, it was proposed that this particular stress protein may provide a role in cellular protection (Maines, 1988; Stocker, 1990), as both haem and HO-1 are found to be highly conserved molecules across almost all forms of life suggesting that they most likely have a necessary and fundamental purpose. Indeed, the case of a boy born with HO-1 deficiency highlighted the importance of this enzyme (Yachie et al., 1999). The child suffered growth retardation, anaemia, tissue iron

deposition, lymphadenopathy, leukocytosis, a marked sensitivity to oxidant injury and unfortunately succumbed to an early death. The child was found to have both intravascular haemolysis and endothelial cell injury. In particular, the lymphoblastoid cell line derived from the patient was shown to have increased sensitivity to haem toxicity (Nagy et al., 2005). There were also fatty streaks and fibrous plaques in the aorta that were reported as a sign of severe atherosclerosis, as well as mesangioproliferative glomerular changes in the kidney (Ohta et al., 2000). Transgenic HO-1 knockout mice have also shown similar endothelial damage as well as hepatic and renal tubular cytotoxicity (Poss and Tonegawa, 1997).

Consequently, HO-1 was seen as necessary for the survival of organisms and experimental evidence supported the hypothesis that this enzyme was protective against oxidative stress (Maines, 1984). Indeed, the beneficial effects of HO-1 have now been described in many diseases (Maines, 1988) (Morse and Choi, 2002). The mechanism by which HO-1 confers this protection has been an area of immense interest, because the discovery of how the cell can protect itself from oxidative stress will lead to new therapeutic avenues in treating and possibly preventing diseases. HO-1 can function as an anti-oxidant by preventing the participation of free haem in pro-oxidant reactions (Keyse and Tyrrell, 1989), but a major area of research has concentrated on the cytoprotective properties of its breakdown products. Haem oxygenase cleaves the α-meso carbon bridge of haem molecules, which is the first rate-limiting step of haem degradation resulting in the production of equimolar quantities of BV IXα, free iron and CO. BV is subsequently converted to bilirubin (BR) by biliverdin reductase (BVR) and free iron is sequestered by ferritin (Figure 1-1).

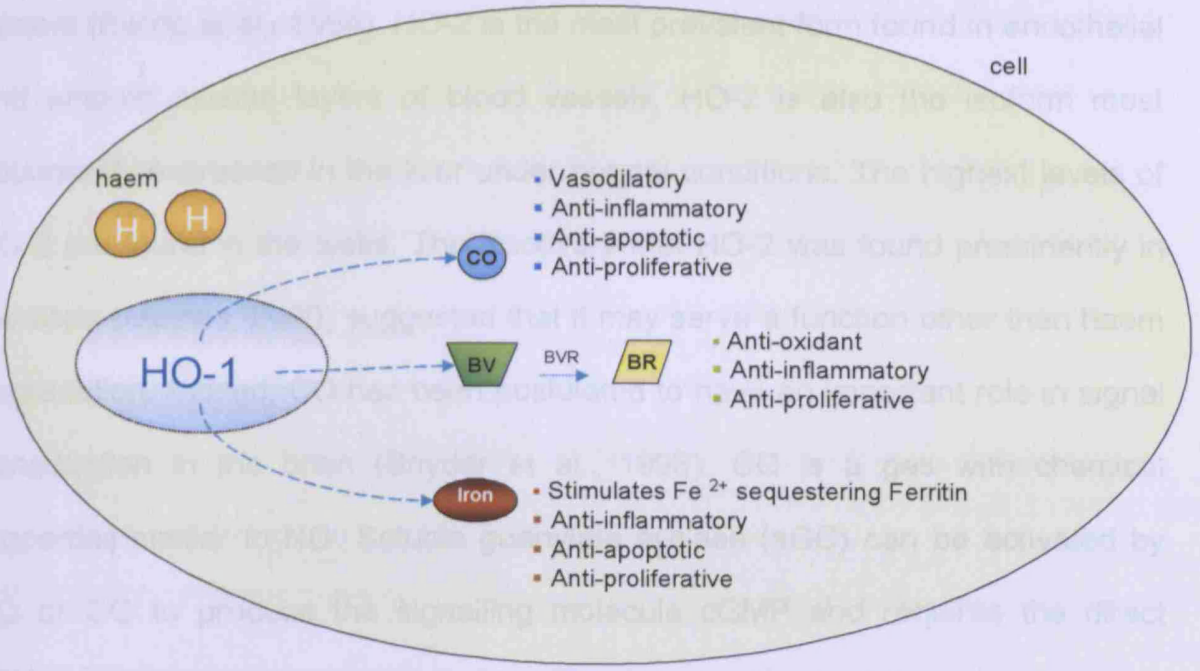


Figure 1-1. The haem oxygenase pathway

Haem oxygenase-1 degrades haem (H) into carbon monoxide (CO), biliverdin (BV) which is converted to bilirubin (BR) through the action of biliverdin reductase (BVR), and iron. The breakdown products can exert various biological actions to protect against cellular damage from oxidative stress.

All three breakdown products, i.e. CO, BR and iron, have been shown to have anti-oxidant properties and are candidate molecules for the anti-inflammatory, anti-apoptotic and anti-proliferative functions of HO-1 (Otterbein and Choi, 2000). There are several isoforms of haem oxygenase, each the product of separate genes. Specifically, HO-1 is the inducible form, HO-2 is a constitutive form and the more recently identified HO-3 is structurally similar to HO-2 but is a less efficient haem catalyst (Maines, 1988). The specific activity of haem oxygenase in different organs varies, with the highest haem oxygenase activity found in the spleen, testes and brain. The spleen is the only organ in which, under normal conditions, HO-1 is the predominant form; however, stressful stimuli will dramatically increase the expression of HO-1 mRNA in the cardiovascular

system (Ewing et al., 1994). HO-2 is the most prevalent form found in endothelial and smooth muscle layers of blood vessels. HO-2 is also the isoform most abundantly expressed in the liver under normal conditions. The highest levels of HO-2 are found in the testis. The discovery that HO-2 was found prominently in the brain (Maines, 2000) suggested that it may serve a function other than haem degradation. Indeed, CO has been postulated to have an important role in signal transduction in the brain (Snyder et al., 1998). CO is a gas with chemical properties similar to NO. Soluble guanylate cyclase (sGC) can be activated by NO or CO to produce the signalling molecule cGMP and requires the direct binding of the gaseous molecules to the haem moiety of guanylate cyclase (Krumenacker et al., 2004). As HO-2 was found highly expressed in neurons it was hypothesized that CO generated by haem degradation could function as an activator of sGC and, therefore, cGMP production. CO has also been found to function as a signal for cGMP production in the cardiovascular system (Motterlini et al., 1998; Sammut et al., 1998).

1.1.2 Carbon monoxide

CO has been shown to be highly protective in several rodent disease models, mimicking the action of HO-1. For example, CO administration has been shown to have protective effects in models of acute lung injury, endotoxic shock and in mouse cardiac xenotransplantation (Otterbein et al., 1999; Yachie et al., 1999);(Sato et al., 2001). HO-1 expression or CO administration can mediate potent anti-inflammatory effects in monocytes and/or macrophages, probably by preventing these cells from inducing tissue injury and by modulating their role in the initiation of immune responses (Sawle et al., 2005; Yachie et al., 1999). CO also exerts other actions which contribute to its anti-inflammatory

effects. Firstly, CO prevents platelet activation and aggregation (Brune and Ullrich, 1987), thereby suppressing thrombosis and the pro-inflammatory response stimulated by activated platelets. CO also down-modulates the expression in macrophages of plasminogen activator inhibitor type 1; this action appears to be crucial for the ability of CO to exert a protective effect in a model of ischemia-reperfusion of the lung (Fujita et al., 2001). Furthermore, CO prevents apoptosis in several cell types including endothelial cells, fibroblasts, hepatocytes and ß-cells of the pancreas (Brouard et al., 2002) (Gunther et al., 2002; Petrache et al., 2000). As widespread apoptosis can exacerbate the deleterious effects of inflammatory reactions, including the prothrombotic stimulus apoptotic endothelial cells provide (Bombeli et al., 1999), the anti-apoptotic effect of CO may have a crucial role in its overall effect. CO has also been shown to have an anti-proliferative effect on smooth muscle cells that contribute to neointimal proliferation associated with inflammatory lesions (Otterbein et al., 2003b). However, it is important not to overlook the toxicity associated with CO, as the gas is lethal at high enough doses. In fact, CO has the ability to bind haemoglobin and myoglobin tightly thus preventing oxygen delivery to tissues and organs, which leads to tissue hypoxia. High concentrations of CO have also been shown to poison the respiratory chain in the mitochondria thereby suppressing ATP generation (Haab, 1990). The search for cellular targets for CO is only just beginning. However, depending on the cell type, one or more key signalling systems are reported to be involved in the response to CO, including guanylyl cyclase-cGMP, potassium channel activation or p38 mitogen-activated protein kinase (MAPK). For example, cGMP but not p38 MAPK is needed for the anti-apoptotic effect of CO in fibroblasts

(Gerard et al., 1993). In contrast, p38 activation is required for the anti-apoptotic effect of CO in endothelial cells but cGMP is not (Brouard et al., 2000). Furthermore, CO has been reported to mediate arterial vasodilation through activation of calcium-dependent potassium channels in rat models (Bolognesi et al., 2007). There are possible targets through which CO can mediate signal transduction, including proteins in which haem functions as a prosthetic group e.g. haemoglobin, myoglobin, guanylyl cyclase, cyclooxygenase, cytochrome p450 oxidase, inducible nitric oxide synthase (iNOS), NADPH oxidase (Taille et al., 2005) and the transcription factors Bach 1 and Bach 2, to which haem groups are bound in certain conditions (Otterbein et al., 2003a). CO could modulate the activity of these haemoproteins because of its ability to bind to the central iron group contained within their haem prosthetic groups. CO binding would then induce conformational changes that can modulate the biological activity of these proteins (Stevenson et al., 2001), in addition CO could also bind to proteins that contain other metal ions, such as zinc, copper and manganese.

1.1.3 Biliverdin and Bilirubin

Biliverdin (BV) is converted to bilirubin (BR) by the action of biliverdin reductase (Singleton and Laster, 1965). The potent anti-oxidant effects of BV and BR are thought to contribute significantly to the overall protective effect of HO-1 (Stocker et al., 1987b). BR is the most abundant endogenous anti-oxidant in mammalian tissues accounting for the majority of the anti-oxidant activity of the serum. BR at low concentrations scavenges reactive oxygen species (ROS) *in vitro*, reduces oxidant-induced cellular injury and attenuates oxidant stress *in vivo* (Clark et al., 2000a; Stocker et al., 1987a; Stocker et al., 1987b). In

addition to providing protection against oxidative stress, BV and BR have been shown to scavenge NO (Kaur et al., 2003) and therefore could also protect cells against uncontrolled NO production or "nitrosative stress". Furthermore, inhibition of NADPH oxidase (Kwak et al., 1991) and protein kinase C (PKC) activity (Sano et al., 1985) by BR has been demonstrated, both enzymes being implicated in angiotensin II-induced vascular injury (Rajagopalan et al., 1996). Both BV and BR have been reported to have the ability to preserve endothelial cell integrity (Sedlak and Snyder, 2004) and prevent endothelial cell death or sloughing to enhance vascular reactivity in diabetic rats (Abraham et al., 2004). In addition, BR has been implicated in reducing oxidative stress in experimental diabetes, partly by increasing the bioavailability of NO needed for endothelial cell integrity (Abraham et al., 2004). In this context, BR mediated inhibition of PKC and NADPH oxidase may be one mechanism by which HO-1 attenuates the diabetes-mediated generation of oxidants and the uncoupling of eNOS. HO-1 derived BR also displays cytoprotective effects in the cardiovascular system (Clark et al., 2000b). Numerous reports indicate that a higher serum BR level is associated with a decrease in the risk for coronary artery disease in humans. Free and albumin-bound BR has also been shown to inhibit oxidation of low-density lipoprotein (LDL) (Neuzil and Stocker, 1994). Interestingly, the recent description of a cycle in which BV, derived from the oxidation of BR, is converted to BR by BVR suggests a mechanism to amplify the anti-oxidant effects of the two bile pigments (Baranano et al., 2002).

1.1.4 Iron

The degradation of haem by HO-1 leading to the release of Fe^{2+} from the core of the haem molecule, results in the rapid expression of the iron

sequestering protein ferritin (see section 1.2.5) as well as an ATPase pump that actively removes intracellular iron from the cell (Ferris et al., 1999). Expression of ferritin was originally reported to be part of the mechanisms by which HO-1 conferred resistance to oxidative stress in endothelial cells (Balla et al., 1992a), as well as mediating the protection seen in a model of hyperoxic lung injury after HO-1 induction by haem (Taylor et al., 1998). Conversely, the potent cytoprotection provided by HO-1 induction in a model of endotoxic shock in rats appeared to be independent of ferritin (Otterbein et al., 1997). The ATPase pump that actively removes intracellular iron from the cell contributes to decreasing the overall intracellular pool of Fe^{2+} and protecting cells from apoptosis (Ferris et al., 1999). The respective contributions of the iron pump versus ferritin to the overall cytoprotective effect of HO-1 are not clear but presumably both mechanisms participate in a crucial manner to the overall homeostatic effects that follow increased HO-1 expression in a variety of stressful situations.

1.1.5 Induction and regulation of HO-1

A number of physiological and non-physiological stressors can stimulate HO-1 expression and activity, including the substrate haem, endotoxin, inflammatory cytokines, long wave ultraviolet A radiation (UVA), hyperthermia and heavy metals (Ryter and Choi, 2002) (Figure 1-2). The primary mechanism of induction is through activation of the *hmox*-1 gene (Alam and Cook, 2003). Investigations into the gene activation process have revealed a multitude of varied mechanisms of gene regulation.

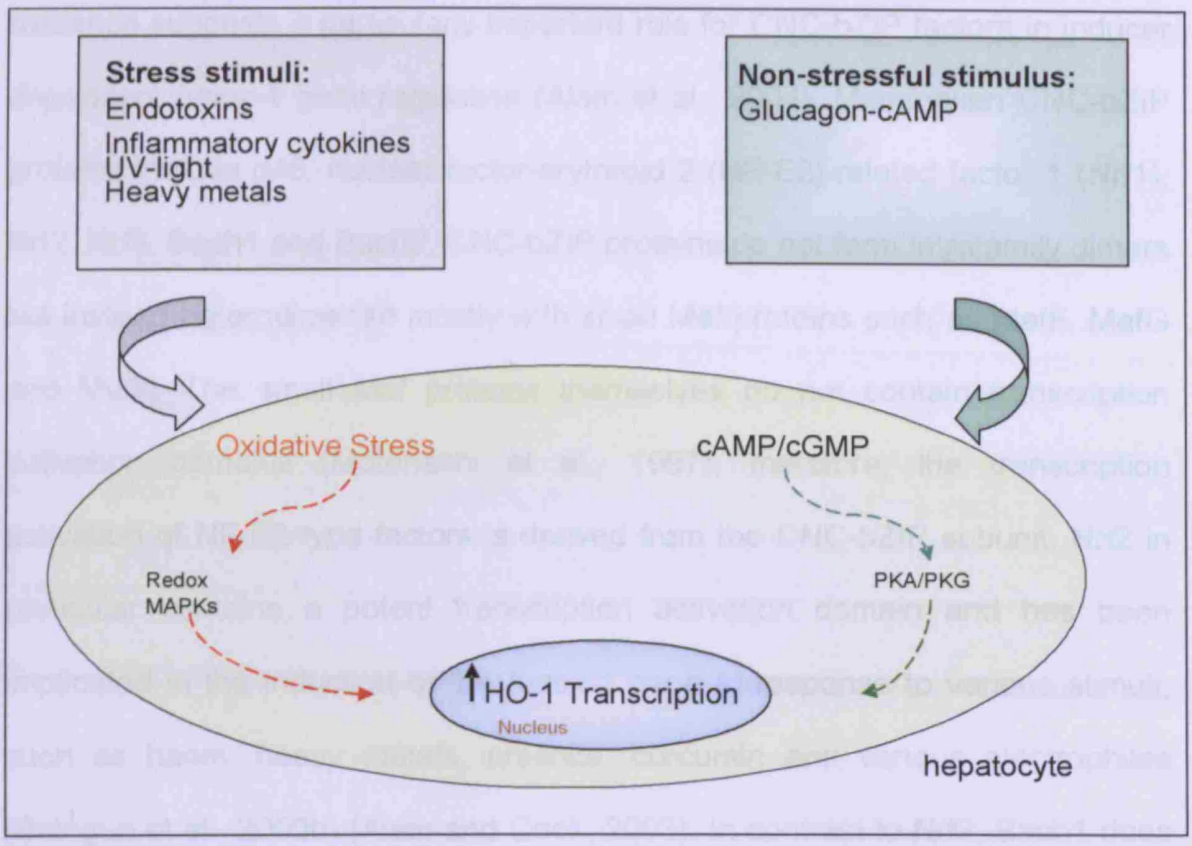


Figure 1-2. Induction of HO-1

Stressful stimuli elicit haem oxygenase-1 (HO-1) induction through activation of mitogen-activated protein kinases (MAPKs), whereas non-stressful stimuli induce HO-1 through increasing cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) production which activate cAMP-dependent protein kinase A or cGMP-dependent protein kinase G (see section 1.1.6).

The best characterized amongst the various *hmox*-1 genes is the mouse *hmox*-1 locus, where analysis has revealed a 10-bp DNA sequence motif called the stress response element (StRE). This motif is found in multiple copies within two upstream enhancer regions and is involved in the mediation of transcription activation in response to almost all HO-1 inducers tested. The StRE are targets of multiple dimeric proteins made up of individual members of the Jun, Fos, CREB, ATF, Maf and Cap'N'Collar-basic-leucine zipper (CNC-bZIP) subclasses of the basic-leucine-zipper superfamily of transcription factors. Increasing

evidence suggests a particularly important role for CNC-bZIP factors in inducer dependent *hmox*-1 gene regulation (Alam et al., 2004). Mammalian CNC-bZIP proteins include p45, nuclear factor-erythroid 2 (NF-E2)-related factor 1 (Nrf1), Nrf2, Nrf3, Bach1 and Bach2. CNC-bZIP proteins do not form intrafamily dimers but instead heterodimerize mostly with small Maf proteins such as MafF, MafG and MafK. The small Maf proteins themselves do not contain transcription activation domains (Motohashi et al., 1997), therefore, the transcription activation of NF-E2-type factors is derived from the CNC-bZIP subunit. Nrf2 in particular contains a potent transcription activation domain and has been implicated in the induction of the *hmox*-1 gene in response to various stimuli, such as haem, heavy metals, arsenite, curcumin and various electrophiles (Balogun et al., 2003b);(Alam and Cook, 2003). In contrast to Nrf2, Bach1 does not contain transcription activation domains but instead Bach-small Maf dimers are thought to act as repressors when bound to target sequences. Indeed, Ogawa and co-workers have demonstrated that Bach1-mediated repression plays a crucial role in the regulation of HO-1 expression (Ogawa et al., 2001).

Bach1-Maf dimers have been shown to bind to the *hmox*-1 StRE which then results in the repression of a linked reporter gene. Bach1 repressor activity is dominant over the activator function of other StRE binding protein such as Nrf2 under normal conditions, which results in a low level of *hmox*-1 activity (Sun et al., 2002). *In vitro*, it has been demonstrated that Bach1 can bind to haem, and haem binding inhibits the DNA binding activity of Bach1. Therefore, it was proposed that induction of HO-1 expression may be the result of inactivation of Bach1 mediated *hmox*-1 gene repression by increased levels of cellular haem (Ogawa et al., 2001). Indeed, Ogawa's group also demonstrated that, *in vivo*,

haem promoted the displacement of Bach1 from the *hmox*-1 enhancer resulting in gene derepression. Displacement of Bach1 from the *hmox*-1 StREs by haem was followed by binding of Nrf2 (probably as Nrf2:small Maf dimer) (Ogawa et al., 2001), suggesting that removal of Bach1 alone is not sufficient to promote optimal *hmox*-1 gene activity and, therefore, high level gene transcription requires the subsequent StRE binding of a protein with activator function. Indeed, Alam and colleagues have demonstrated that Nrf2 is necessary for *hmox*-1 gene induction by haem in rat kidney proximal tubular cells (Alam et al., 2003).

However, HO-1 is induced by many non-haem inducers, which are also known to act via the StRE. Due to the varied structure and nature of these inducers it is unlikely that they would all bind exclusively to Bach1 and inhibit its activity. One possible model put forward for the action of these inducers was that they may act indirectly by increasing the intracellular concentration of inhibitory haem molecules by promoting the release of haem from haemoproteins or mobilising a "free" haem pool. However, this model has been challenged, and evidence has been put forward to offer different mechanisms of action (Alam et al., 2004). For example, cadmium, a potent inducer of HO-1, has been shown to promote the export of Bach1 from the nucleus, as well as the nuclear export of Bach2, suggesting that cadmium can alleviate repression of *hmox*-1 by Bach1 or Bach2 (Suzuki et al., 2003). Cadmium, like haem, can stimulate Nrf2 expression by inhibiting its degradation (Stewart et al., 2003), resulting in relative nuclear abundance of StRE activators (e.g. Nrf2:small Maf dimers) compared with StRE repressors (e.g. Bach-small Mac dimers) and *hmox*-1 gene activation. The overall effect after the exchange of factors at the

StRE is an increase in the rate of *hmox*-1 transcription. A reduction in the stress environment by termination of exposure or sequestration, cellular extrusion or metabolic inactivation of the stressor will result in reversal of these processes and a return to the original state of the unstimulated cell (Alam et al., 2004).

The number of StRE, their location and sequence are highly conserved between the mouse and human *hmox*-1 genes, which infers that the StRE pathway is important in the induction of human HO-1 expression. However, there is limited analysis to date of the human StRE pathway, and further investigation will be required to elucidate the importance of this regulatory system (Alam et al., 2004).

1.1.6 HO-1 and signal transduction

Whereas haem acts as a ligand and interacts directly with DNA binding proteins (Bach1), this is not the mechanism of action for the majority of the HO-1 inducers. Increasing evidence suggests that the majority of inducers increase expression of HO-1 by activating one or more signalling cascades that eventually converge on transcription factors or repressors to regulate the activity of the *hmox*-1 gene (Alam et al., 2004). For example, protein kinases mediate hepatic HO-1 induction by cyclic nucleotides. cAMP and cGMP are second messengers which play an important role in intracellular signal transduction of various hormones and extracellular stimuli. Increases in these cyclic nucleotides activate downstream-dependent protein kinase A (PKA) and cGMP-dependent protein kinase G. cAMP has been shown to induce HO-1 expression in rat hepatocytes via the PKA signalling pathway (Immenschuh and Ramadori, 2000). PKA-dependent, cAMP mediated HO-1 induction has also been seen in vascular smooth muscle cells (Durante et al., 1997). The

signalling gas NO induces HO-1 expression in many tissue and cell types (Motterlini et al., 1996b; Motterlini et al., 1996c; Motterlini et al., 1996a; Naughton et al., 2002b). As NO potently stimulates soluble guanylate cyclase, with a resulting high level of cGMP production, it can be proposed that cGMP may serve as a second messenger for NO-mediated *hmox*-1 gene activation. Indeed, cGMP treatment on its own has been shown to increase *hmox*-1 transcription via activation of PKG in hepatocytes and endothelial cells (Immenschuh et al., 1998; Polte et al., 2000). However, other reports do not confirm cGMP as a mediator of HO-1 induction by NO (Chen and Maines, 2000; Hartsfield et al., 1997; Motterlini et al., 1996a), suggesting the role of NO in haem oxygenase gene modulation is complex and requires further investigation. A more detailed description of the interaction of NO and haem oxygenase is given in sections 1.6.8-11.

Mitogen-activated protein kinases (MAPKs) have also been shown to have an important role in stress mediated cellular responses. MAPKs are a family of serine-threonine protein kinases which are involved in the transduction of extracellular signals, leading to a wide variety of intracellular responses such as gene expression, cell proliferation and apoptosis. The three major subfamilies of MAPKs that have been identified are: extracellular regulated signal kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 kinases. MAPKs are activated by phosphorylation of their threonyl and tyrosyl residues by MAPK kinases (MKKs) which in turn are phosphorylated and activated by upstream MKK kinases (MKKKs) (Kyriakis and Avruch, 2001). There have been divergent results reported for the stress-dependent stimulation of the *hmox*-1 gene activity in hepatic cells from different species. For example, Kong and co-workers have

implicated several MKKKs in arsenite mediated Nrf2 dependent HO-1 up-regulation in human Hep-G2 hepatoma cells (Yu et al., 2000), the response of which is in part dependent on the activity of JNK1 and its upstream kinase MKK4, but does not require p38 or its upstream kinase MKK3. In contrast, arsenite-induced HO-1 expression in rat hepatocytes requires both the JNK and MKK3/p38 pathways (Kietzmann et al., 2003). Therefore, there are clearly species-specific differences in the involvement of MAPK signalling cascades during stress-dependent induction of hepatic HO-1 expression.

UVA radiation (320-380nm) is also known to induce HO-1. UVA radiation is an oxidizing carcinogen whose biological effects are dependent on oxygen. Singlet oxygen specifically is thought to be an early intermediate in the induction of the *hmox*-1 gene. The next steps appear to involve lipid oxidation products and metabolites (Basu-Modak and Tyrrell, 1993). Oxidized lipids and oxidized membrane phospholipids are potent inducers of HO-1 induction; however, some evidence suggests that enzymatic processing of UVA damaged lipids to prostaglandins through the phospholipase A2/cyclooxygenase pathway is also critical to UVA activation (Basu-Modak et al., 1996), as prostaglandins are strong inducers of HO-1 (Grether-Beck et al., 2000). UVA radiation can also, through generation of lipid intermediates, potentially involve signalling pathways. For example, reports indicate that UVA activates each of the three major classes of MAPKs in both fibroblasts and epidermal cells under certain conditions (Klotz et al., 1999a; Le Panse et al., 2003; Silvers et al., 2003). Furthermore, in human fibroblasts it has been postulated that *hmox*-1 is activated through various kinases (Klotz et al., 1999b). UVA has also been shown to activate protein kinase C which through its anti-apoptotic properties

could be linked to the anti-apoptotic properties of HO-1 (Matsui and DeLeo, 1990).

1.1.7 HO-1 as a therapeutic target

The potent protective properties of HO-1 lead to the question of whether induction of HO-1 can be used clinically. In addressing this question it is important to consider the polymorphism identified in the human HO-1 gene promoter (Kimpara et al., 1997), which regulates the magnitude of HO-1 response to a given stress signal. This polymorphism appears to be of functional significance in those patients with long GT repeats in the gene promoter region of HO-1 which elicit low HO-1 responses. Conversely, patients with short GT repeats in the HO-1 gene promoter elicit a high HO-1 response, and are associated with a reduced likelihood of restenosis after angioplasty (Exner et al., 2001). Therefore, the induction of HO-1 in certain patients with low responses would be ineffective therapeutically. However, the administration of HO-1 biproducts could also have potential therapeutic applications and provide an alternative approach to liberating the protective effects of HO-1 in a clinical setting.

Until recently, the use of CO as a therapeutic tool may have appeared to be an unattractive prospect due to its toxicity. However, studies using relatively low doses of inhaled CO (250 ppm for just 1 to 24 h) pre-operatively in rodent and pig models have produced beneficial results in attenuating post-operative ileus without producing unwanted side-effects (Moore et al., 2005). If similar beneficial results are seen in humans there may be a potential for the use of CO therapeutically, for example in the pre-treatment of patients before vascular surgeries to prevent restenosis or the treatment of an organ donor and/or the

organ to suppress ischaemia-reperfusion injury and to prolong allograft survival in transplantation procedures (Otterbein et al., 2003a). Indeed, HO-1-derived CO or inhaled CO have both demonstrated suppression of arteriosclerotic lesion development associated with chronic rejection of transplanted organs in rodent models (Otterbein et al., 2003b; Sato et al., 2001). However, the use of CO gas as a therapeutic agent has significant obstacles. The gaseous nature of the molecule underlies the difficulty in delivering CO efficiently and securely to target cells (Alberto and Motterlini, 2007). The recent identification of CO-releasing molecules (CO-RMs), a novel class of compounds that can carry and transport CO into a biological environment (Motterlini et al., 2003), offers an intriguing alternative to CO delivery through inhalation (see section 1.1.8 for more details). Accumulating evidence has demonstrated that CO-RMs can be used therapeutically to suppress inflammation and protect against oxidative stress, ischaemia-reperfusion injury, allograft rejection and myocardial infarction in animal models (Motterlini et al., 2005b). The potential use of CO-RMs in human patients would offer an accurate and safe way to deliver the therapeutic properties of CO. However, further studies are essential for ascertaining the optimal concentration of CO to be administered, as well as the length and frequency of CO treatment required to have a beneficial effect for a given condition.

Administration of BV or BR provides an additional strategy to exploit the beneficial effects of HO-1 induction, and could be used as an independent treatment regimen or in combination with CO therapy. BR is a potentially toxic and insoluble molecule, and in high doses can contribute to cytotoxicity, for example, hyperbilirubinaemia is responsible for diseases such as neonatal

jaundice and kernicterus (Kapitulnik, 2004). However, investigators demonstrated that micromolar levels of exogenous BR minimised the effects of ischaemia-reperfusion injury in rat liver or kidney transplantation models and was equally as effective as HO-1 induction in protection against oxidative stress (Kato et al., 2003) (Adin et al., 2005). These results suggest that BR administration may provide organ protection during graft harvest, where its anti-oxidant properties could protect against ischaemia and oxidative injury. Unlike BR, BV is a soluble and non-toxic compound; but it is rapidly converted to BR by BVR, and several investigators have supplied exogenous BV as a safer means of harnessing the anti-oxidative properties of BR. For example, BV administration resulted in increased survival of mouse cardiac allograft and transplanted rat small bowel (Yamashita et al., 2004);(Nakao et al., 2004). Furthermore, co-administration of BV and CO resulted in significantly more protection of organ function and survival in rat kidney and heart transplant models than observed with BV alone (Nakao et al., 2005). Therefore, BV or BR in combination with CO could potentially provide protection from oxidative damage in human organ harvesting and transplantation. However, further studies are required to determine the optimal dose and timing of BV or BR delivery, necessary for therapeutic effect in clinical settings.

1.1.8 CO-releasing molecules

Until 10 years ago, CO was considered to be nothing more than a waste molecule produced in living organisms with little functional use. The toxic effects of high levels of CO have been well documented and are primarily due to its binding to important biological haemoproteins such as haemoglobin and cytochromes which are essential for O_2 transport and mitochondrial respiration.

However, in the last decade a new appreciation of the versatile properties of CO as a signalling mediator and regulator of essential physiological processes has emerged. Endogenous generation or exogenous administration of low doses of CO has been demonstrated to have potent vasodilatory, anti-inflammatory, anti-proliferative and anti-apoptotic effects (Motterlini et al., 2003). Therefore, to harness these potentially therapeutic effects of CO, the search for "CO-releasing molecules" (CO-RMs) was initiated in our laboratory. The promise of such compounds was to carry and deliver a therapeutic but potentially toxic molecule, controllably and safely into biological systems.

The initial exploration into CO-RMs identified two metal carbonyl complexes, $Mn_2(CO)_{10}$ (CORM-1) and $[Ru(CO)_3Cl_2]_2$ (CORM-2), which were observed to liberate CO. Significantly, the biological activity of these CO-RMs was confirmed in isolated rat heart or aortic vessel models, where both compounds produced powerful vasodilation (Clark et al., 2003);(Motterlini et al., 2002a). However, these early CO-RMs were poorly soluble in water which would be a limitation to potential pharmaceutical development.

Two water-soluble CO-RMs were then synthesised: tricarbonylchloro(glycinato)ruthenium(II) (CORM-3) and sodium boranocarbonate (CORM-A1), which have demonstrated the most encouraging results (Alberto and Motterlini, 2007). CORM-3 and CORM-A1, despite diverse chemical structures and differing rates of CO release, have elicited significant therapeutic effects in a variety of experimental models and will be discussed in more detail in the following sections. Importantly, the use of inactivated CO-RMs (iCORMs), which are the CO-RM molecules depleted of CO, as negative controls in experimental settings have confirmed that it is the effect of CO

modulating physiological functions and not another component of the CO-RM molecule (Motterlini et al., 2003).

1.1.8.1 Pharmacological actions of CORM-3

CORM-3 is a metal carbonyl complex (see section 2.3.1 for synthesis), which can rapidly release CO into physiological buffers. It has a half-life of approximately 3.6 min at 37°C and pH 7.4. CORM-3 has developed into a versatile research tool and has been shown to liberate CO *in vitro*, *ex vivo* and *in vivo* in biological models (Motterlini et al., 2003). The vaso-active properties of CORM-3 have been reported in a rat isolated aortic ring model, where CORM-3 produced significant vasodilation (Foresti et al., 2004). The role of CORM-3 in attenuating inflammatory responses has also been reported in various experimental models. For instance, CORM-3 significantly suppresses the LPS-mediated inflammatory response in cultured macrophages (Sawle et al., 2005), as well as LPS or interferon-gamma-induced inflammation in BV2 microglial cells (Bani-Hani et al., 2006). CORM-3 also showed therapeutic results in *in vivo* models of acute inflammation (e.g. acute peritonitis in mice), as well as reducing endothelial adhesion molecule expression and subsequent leukocyte-endothelial interactions (Urquhart et al., 2007). These studies suggest that CORM-3 can be used as an effective tool to modulate inflammation.

The cardio-protective properties of CORM-3 have been another major area of interest for investigators. CORM-3 has been shown to protect myocardial cells and isolated hearts against ischaemia-reperfusion injury as well as cardiac transplant rejection in mice (Clark et al., 2003). In further studies, CORM-3 was reported to limit ischaemia-reperfusion injury in an *in vivo* model of cardiac infarction in mice (Guo et al., 2004). More recently, pre-

treatment of isolated rat hearts with CORM-3 was found to reduce infarct size, incidence of ventricular fibrillation and tachycardia (Varadi et al., 2007) and administration of CORM-3 was found to induce late pre-conditioning against myocardial infarction in an *in vivo* mouse model (Stein et al., 2005). In addition, the positive inotropic effects of CORM-3 in an isolated perfused rat heart model were recently described (Musameh et al., 2006). This mounting evidence suggests that CO offers powerful cardio-protective effects and CORM-3 may provide an effective means of delivery of this therapeutic agent.

CORM-3 has also been found to be beneficial to kidneys, as it protected renal epithelial cells against cisplatin induced nephrotoxicity, as well as preventing cisplatin-mediated renal dysfunction in a rat model of renal failure (Tayem et al., 2006). Furthermore, isolated rabbit kidneys flushed with CORM-3 followed by cold ischaemia elicited at reperfusion an increased glomerular filtration rate, and sodium and glucose re-absorption compared to controls (Sandouka et al., 2006). These data corroborate the potent cytoprotective properties of CO and CORM-3, and also highlight the therapeutic potential of this molecule in a clinical setting for harvesting and transplantation of organs.

1.1.8.2 Pharmacological actions of CORM-A1

CORM-A1 differs from the original CO-RMs in that it does not contain a transitional metal carbonyl but a carboxylic acid coordinated to a boron atom (see section 2.3.2 for synthesis). It possesses a much slower rate of CO release compared to CORM-3, with a half-life of 21 min at 37°C at pH 7.4 (Motterlini et al., 2003). In parallel experiments to those undertaken with CORM-3, the vasorelaxant and hypotensive properties of CORM-A1 have been demonstrated in aortic isolated ring and *in vivo* models, respectively (Motterlini

et al., 2005c). CORM-A1 also elicited significant coronary vasodilation in rat isolated perfused heart models (Musameh et al., 2006), adding to the growing evidence that CO released from CO-RMs has potent cardio-protective actions.

The vasodilatory actions of CORM-A1 were further explored in an *in vivo* mouse kidney model, where CO liberated from CORM-A1 resulted in increased renal blood flow and decrease in vascular resistance (Ryan et al., 2006). The cytoprotective effects of CORM-A1 were also demonstrated in the kidney, where flushing kidneys with CORM-A1 before cold storage produced protective effects on renal function similar to those observed with CORM-3 (Sandouka et al., 2006). Recent work has explored the cerebroprotective effects of CORM-A1, where systemic administration of CORM-A1 was importantly shown to deliver CO to the brain in newborn pigs (Zimmermann et al., 2007). The authors reported that CORM-A1 elicited the vasodilatory and cytoprotective effects of CO in the cerebral circulation and resulted in protection of the neonatal brain from cerebral vascular injury from epileptic seizures. These are intriguing results and emphasize the potent cytoprotective and vaso-active properties of CO delivered by CORM-A1.

1.2 Haem

Haem is essential for the function of all aerobic cells. It is a complex of iron with protoporphyrin IX and is used as a prosthetic group in many haemoproteins (e.g. haemoglobin, cytochromes and guanylate cyclase). Haem also has a role in the control of protein synthesis and cell differentiation. Due to its toxicity and insolubility, the intracellular level of uncommitted haem is maintained at a very low concentration ($<10^{-9}$ M). Its levels are controlled tightly by balancing its synthesis via the enzyme 5-aminolevulinic acid (ALA) and

catabolism by the haem oxygenase enzyme, respectively.

1.2.1 Haem synthesis

The synthesis of haem requires the presence of eight enzymes, four of which are found in the cytoplasm and four in the mitochondria (Figure 1-3). The first step takes place in the mitochondria where ALA synthase catalyzes the condensation of succinyl coenzyme A (CoA) and glycine to form 5-aminolevulinic acid. The next four steps in the haem biosynthetic pathway take place in the cytosol, where ALA dehydratase converts 2 molecules of ALA to a monopyrrole porphobilinogen (PBG). This is followed by two subsequent enzymatic steps which convert 4 molecules of PBG into the cyclic tetrapyrrole uroporphyrinogen III, which is then decarboxylated to form coproporphyrinogen III. The final three steps require a return to the mitochondria (Ponka, 1999). In the final step of haem synthesis, ferrochelatase catalyzes the insertion of iron into the protoporphyrin IX ring to form protohaem. Intracellular iron regulates the expression of ferrochelatase. Iron has to be in the ferrous form to act as a substrate of the ferrochelatase reaction in biological systems; therefore, reduction of ferric iron to ferrous is necessary to produce haem. Hence, the return of the haem biosynthetic pathway to mitochondria from cytoplasm is supposedly due to the requirement of a considerable amount of ferrous iron for the final step in haem synthesis (Ponka, 1999).

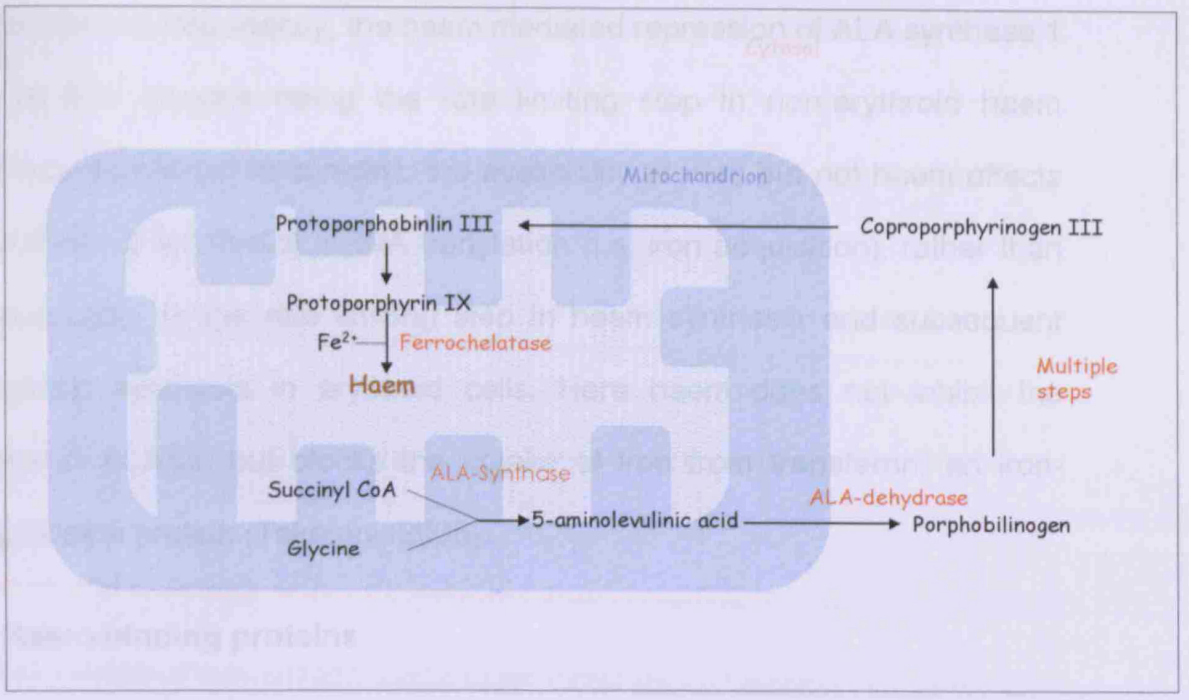


Figure 1-3. Haem synthesis

ALA-synthase catalyses the condensation of succinyl coenzyme A (CoA) and glycine to 5-aminolevulinic acid in the mitochondrion. Subsequent steps in the haem synthetic pathway are carried out in the cytosol until the production of coprophrinogen III, which returns to the mitochondrion for the final steps of haem synthesis including insertion of iron (Fe^{2+}).

ALA synthase has an important role in the regulation of haem synthesis. The enzyme is present in a variety of cells, with high levels found in bone marrow cells and the liver, where it is inducible by chemical treatments or physiological stimuli. ALA synthase is the rate-limiting enzyme of haem biosynthesis in liver and is negatively regulated by haem (Sassa, 1988). There are two isoforms of the enzyme, one of which is ubiquitously expressed (ALA synthase 1) and the other specific to erythroid cells (ALA synthase 2). The mechanism by which its expression in the liver and other non-erythroid tissue is controlled differs from that occurring in haemoglobin synthesizing cells. ALA synthase1 is regulated by haem, in that haem can suppress its transcription by decreasing the half-life of its mRNA or by blocking translocation of ALA synthase 1 precursor protein into

the mitochondria. Importantly, the haem mediated repression of ALA synthase 1 results in this enzyme being the rate limiting step in non-erythroid haem biosynthetic pathways. In contrast, the availability of iron, but not haem affects ALA synthase 2 (erythroid) mRNA translation (i.e. iron acquisition), rather than ALA production, is the rate limiting step in haem synthesis and subsequent haemoglobin synthesis in erythroid cells. Here haem does not inhibit the synthesis of ALAS2, but blocks the uptake of iron from transferrin, an iron-binding plasma protein (Taketani, 2005).

1.2.2 Haem-binding proteins

As well as the breakdown of haem by HO-1, the intra- and extracellular transport of haem also helps to prevent its cytotoxicity. Extracellular haem present in plasma can be taken up by many different cells including hepatocytes; however, mechanisms of haem uptake by these cells are poorly understood. Due to its low solubility in aqueous solutions, haem requires specific extracellular and intracellular proteins as transient carriers. The haem binding proteins in blood and culture medium are serum proteins such as albumin and haemopexin (Taketani, 2005). Haem is toxic in free form but it becomes less toxic or non-toxic in a bound state (with protein). Haemopexin (Hx) is a haem-binding protein and reduces the toxic effect of free haem by forming a complex. It is a serum glycoprotein (60kDa) which binds to haem with high affinity (Delanghe and Langlois, 2001), and plays the role of transporting haem in the circulation. During haemolysis, free vascular haemoglobin can be rapidly converted into methaemoglobin, which liberates the incorporated haem group easily. Hx binds free vascular haem and transports it to the liver for degradation in the reticuloendothelial system (Muller-Eberhard and Fraig,

1993). Two processes of Hx-mediated haem uptake are detected in isolated hepatic parenchymal cells. The first one, defined as the specific transport system, is saturable, has a high haem affinity and exhibits the characteristics of the receptor mediated system *in vivo*. The second uptake system of hepatocytes, normally termed selective, also requires metabolic energy but has a lower affinity for haem and is not readily saturable. This selective haem uptake requires the presence of Hx. The haem-Hx complex binds to its receptor on the liver parenchymal cell plasma membrane and haem is translocated inside the cell, while the apo-haemopexin is released back into the circulation. The toxic effects of haemin are found to be inhibited by Hx (Smith and Morgan, 1981).

Albumin complexes with haem can also prevent the toxic effects of extracellular haem in blood plasma. The haem-albumin complex (methaemalbumin) can transport haem to the parenchyma cells of the liver; however a large proportion of the bound albumin-bound haem is transferred to haemopexin due to its higher affinity for the molecule (Morgan et al., 1976; Smith and Morgan, 1981). Therefore, in normal human subjects, only when Hx is depleted by repeated haem injection does haem bind solely to albumin to produce high methaemalbumin levels (Sears, 1970). Methaemalbumin is an abnormal component of blood plasma in certain diseases and is associated with excessive haemolysis. Under normal conditions, human serum albumin inhibits the toxic effects of haemin by 50-60%. For example, albumin can extract haemin trapped in red blood cell membranes thereby protecting them against the harmful effects of haemin. However, under pathological conditions such as haemolysis, greater accumulation of haemin in red blood cells reduces the

lifespan of the red blood cell (Shaklai et al., 1985).

Recent findings have shown that lipocalin alpha-1 microglobulin (alpha-1 m) binds haem, and after cleavage of a C-terminal tetrapeptide, initiates haem degradation (Larsson et al., 2004). This suggests the protein functions as a haem scavenger. Alpha-1 m, either in plasma or in purified form displays concentration-dependent binding to haem. An interesting role for glutathione (GSH) in counteracting haem-mediated toxicity is described below. Reduced GSH protects cells or tissues from oxidative stress due to its anti-oxidant nature. GSH maintains the redox state of protein-thiol moieties and reduces the noxious hydrogen and lipid peroxides. In addition, GSH offers protection against haem-induced oxidative stress by degrading haem directly and scavenging reactive oxygen species generated by haem. Studies with intact erythrocytes indicate that haem interacts with the membrane of erythrocytes and GSH probably protects erythrocytes from haem-induced oxidative stress by degrading the membrane associated haem (Atamna and Ginsburg, 1995).

In biological systems there are also proteins that can bind selectively free haem. They are known as haem binding proteins (HBP) and reduce the toxic effects of haem by complexing with it. A specific haem binding protein (HBP23) has been purified from rat liver cytosol using haem-affinity chromatography. It is present in the cytosol of liver, kidney, spleen, small intestine and heart with the liver showing the highest content (Iwahara et al., 1995). HBP23 has been proposed to be an intracellular haem transporter i.e. a vehicle from cell membrane to cell (Immenschuh et al., 1995).

1.2.3 Free haem detoxification systems

Haem detoxification is a protective mechanism for the survival of cells

against the pro-oxidant environment created by haem. There are several detoxification-defence mechanisms existing in humans that can be divided into haem oxygenase systems (HO-1, HO-2 and HO-3) and haem oxygenase-independent systems (haemopexin, albumin). As described earlier, haem oxygenase is a haem-degrading enzyme and plays a significant role in protecting cells from haem-induced oxidative stress. The haem oxygenase-independent systems detoxify free haem by forming non-toxic haem-complexes (e.g., haemopexin, albumin, haem-binding protein), by inducing its degradation (e.g., GSH, xanthine oxidase, NADPH-cytochrome P-450 reductase) or by scavenging free redox-active iron (ferritin) released after haem catabolism.

Ferritin is a ubiquitous and highly conserved iron-binding protein. Its importance in sequestering free iron is highlighted by the fact that the toxicity of haem is attributable to the iron released after its degradation. The liberated iron can participate in the generation of reactive oxygen species, which can directly damage DNA, lipids and proteins leading to profound cellular toxicity (Balla et al., 1991). Therefore, ferritin offers a counterbalance to haem oxygenase-dependent haem iron release by binding and storing iron in a form less likely to result in oxidative injury. Indeed, studies have demonstrated endothelial cells exposed to haem up-regulate the synthesis of haem oxygenase and ferritin, and ferritin offers cytoprotection against iron-driven oxidant damage (Balla et al., 1992a).

Haptoglobin (Hp) a serum glycoprotein is a potent haemoglobin scavenger and offers protection against haem toxicity associated with free haemoglobin. For instance, liberated haemoglobin can promote the accumulation of hydroxyl radicals and harmful reactive oxygen species.

Therefore, plasma Hp can be regarded as a major anti-oxidant, protecting cells from damage such as haemoglobin-driven lipid peroxidation. Haemoglobin released from erythrocytes into the circulation by intravascular haemolysis binds immediately to Hp and forms stable Hp-haemoglobin complexes which are transported to the reticuloendothelial system (Wagener et al., 2003), where as the haemoglobin-scavenger receptor CD163 mediates the endocytosis of Hp-haemoglobin complexes and thereby counters the oxidative tissue damage after haemolysis (Philippidis et al., 2004). CD163 is expressed in the monocyte-macrophage system and mediates the endocytosis of Hp-haemoglobin complexes formed on red blood cell haemolysis, and thereby leads to lysosomal degradation of the ligand protein and metabolism of haem by cytosolic haem oxygenase (Moestrup and Moller, 2004).

Although the haem oxygenase system is the principal pathway used to eliminate free haem, other minor haem degradation pathways are also present. Haem can be degraded by haem oxygenase-independent pathways such as NADPH-cytochrome p450 reductase, which can catalyze oxidative degradation of haem molecules. Unlike the haem oxygenase pathway, the major products do not include BV and only a small amount of CO is produced (Docherty et al., 1984). In the cytosol, xanthine oxidase can also catalyse the degradation of haem to non-BV type compounds (Cantoni et al., 1981).

1.2.4 Haem receptors

Haemopexin-haem activates HO-1 after being taken up by cells in a receptor mediated manner (Alam and Smith, 1992). Haemopexin receptors have been found in various tissues such as rat liver and human placenta, but the properties of these receptors are still unknown (Ren and Smith, 1995;

Taketani et al., 1987). Along with haemopexin receptors, haem receptors have been reported on the surface of MEL cells, Caco cells and hepatocytes (Galbraith et al., 1985; Galbraith, 1990; Uc et al., 2004). It has been suggested by some groups that haem enters these cells via an internalization process mediated by a haem-receptor (Galbraith et al., 1985; Galbraith, 1990; Uc et al., 2004). However, due to the lipophylic nature of haem, it could be proposed that haem may readily incorporate into endothelial cell membranes. A new clue on the mechanism of haem transport was provided recently by Shayeghi and co-workers (Shayeghi et al., 2005) who described the presence of a haem transporter that is expressed in the apical region of epithelial cells in the mouse duodenum. The transporter was identified as the membrane protein named HCP1 (haem carrier protein 1) which mediated haem uptake by cells and was regulated by iron. Thus, it was suggested that the transport of haem into cells requires specific membrane bound transporters rather than passive diffusion and incorporation into cell membranes (Shayeghi et al., 2005).

1.2.5 Haem in the circulation: effects on the vascular endothelium

Red blood cells which contain haem concentrations of 20 mmol/l can undergo unexpected lysis and release free haemoglobin into the circulation. Haemoglobin in its reduced form (ferro or oxyhaemoglobin) is relatively harmless to cells; conversely, oxidized haemoglobin (ferri or methaemoglobin) greatly amplifies oxidant-mediated endothelial cell injury. This is due to methaemoglobin's ability to readily release its haem moieties, which can then be readily incorporated into cell membranes and serve as a source of highly toxic iron (Balla et al., 2005). However, oxyhaemoglobin can be readily oxidized to methaemoglobin in the presence of oxidants derived from inflammatory cells;

for example, activated polymorphonuclear cells (PMNs) can rapidly cause oxidisation of oxyhaemoglobin to methaemoglobin (Balla et al., 1993), whilst NO is another candidate for the production of methaemoglobin. In addition, the reaction between oxyhaemoglobin and NO not only produces methaemoglobin but also results in the reduction of NO bioavailability, thus producing deleterious effects such as pulmonary hypertension and end-organ injury (Gladwin et al., 2004). Nevertheless, the initial release of haem from methaemoglobin can be inhibited by the formation of a complex with the haemoglobin-binding protein haptoglobin and any released haem can bind to haemopexin, thereby limiting the deleterious effects of methaemoglobin on the endothelium (Bunn and Jandl, 1968).

However, severe haemolysis that occurs in pathological states such as SCD, ischemia reperfusion and malaria results in high levels (up to 20 μM) of free circulating haem (Arruda et al., 2004). Under these conditions, the physiological mechanisms of removing free haem from the circulation are severely impaired. Because of its lypophilic nature, haem will cross cell membranes leading to non-specific haem uptake and haem-catalyzed oxidation reactions, thereby enhancing cellular oxidant damage (Reiter et al., 2002). In other words, when large amounts of free haem accumulate, the haem detoxification systems are overwhelmed and haem can exert its damaging effects.

The vasculature, and in particular the endothelium, may be at the greatest risk from exposure to toxic free haem. Vascular endothelial cells have adapted to oxidative stress due to their constant exposure to haem via the anti-oxidant and cytoprotective actions of haem oxygenase and ferritin. Haem and

various other stimuli have been shown to cause the induction of HO-1 and ferritin in endothelial cells and other cell types (Balla et al., 2005). HO-1 could serve as a provider of intracellular iron from haem; the iron in turn may drive the production of ferritin synthesis. Another proposed mechanism is that haem itself may induce ferritin production directly by increasing RNA translation (Lin et al., 1990). Haemoglobin has also been shown to enhance HO-1 and ferritin gene induction in vascular endothelial cells, though conversion of oxyhaemoglobin to methaemoglobin is required for this process, as methaemoglobin can easily release its haem (Balla et al., 1992b; Balla et al., 1993). Brief exposure to haem or haemoglobin can result in increased oxidant-mediated endothelial cytotoxicity, but longer duration of exposure induces an adaptation and protection in cells from further oxidant challenge. This protection is paralleled by the synthesis and accumulation of large amounts of HO-1 and ferritin. The protection provided by ferritin in endothelial cells is attributed to iron storage or ferroxidase activity of ferritin (Balla et al., 1993). Under aerobic conditions ferroxidase activity catalyses the oxidation of ferrous iron to ferric iron, which allows intracellular storage of iron in biological systems. The protective function of ferritin may also be linked to its high affinity for ferrous (Fe^{2+}) iron formed by superoxide (O_2^-) or endogenous reductants and, although, it's ferroxidase activity stores and inactivates iron in the ferric form, it is the reduced form of iron that can fuel production of the hydroxyl radical. Therefore, increased cellular ferritin may compete for ferrous iron, thereby reducing the pool of catalytically active iron species. Catabolism of haem by haem oxygenase may help to eliminate the membrane permeable form of iron from cells, since the resultant non-haem iron would be potentially hazardous unless sequestered by ferritin

(Balla et al., 1992a). The cytoprotective nature of haem oxygenase and ferritin have been confirmed in various animal models (Balla et al., 2003).

1.2.6 Haem and oxidative stress

The presence of free haem causes toxic effects to tissues and organs through oxidative stress and by generating reactive oxygen species, the redox-active iron contained in haem playing a central role in haem-mediated toxicity. Haem-induced cytotoxicity has been shown to impair the function of kidney (Nath et al., 2001b), neurons (Goldstein et al., 2003), cardiac cells (Bhoite-Solomon et al., 1993), hepatocytes (Kim et al., 2004) and peripheral leukocytes (Tsuji et al., 1993). In addition, iron-derived reactive oxygen species are implicated in the pathogenesis of many vascular disorders including atherosclerosis, vasculitis and reperfusion injury (Balla et al., 2005). Intracellular haem proteins are an abundant source of redox-active iron and free haem damages lipids, proteins and DNA (Kumar and Bandyopadhyay, 2005). For instance, haemin in the aqueous phase will form aggregates in cell membranes and promote oxidation, which leads to enhancement of permeability and membrane dysfunction. In addition, oxidation of membrane components may promote cell lysis and death (Schmitt et al., 1993). Exposure of endothelial cells to haem also greatly potentiates cell killing mediated by PMNs (Balla et al., 2003). These cells usually marginate along endothelial cells in the presence of inflammation. Haem has been shown to induce PMN activation (Graca-Souza et al., 2002) as well as enhance endothelial cell adhesion molecule expression (Wagener et al., 1997), thus promoting PMN adhesion and stimulating inflammation. In addition, haemin (the Fe^{3+} oxidation product of haem) has been shown to catalyse the degradation of proteins to small peptide fragments, e.g.,

the serum protein HBP93 (Aft and Mueller, 1984). Haemin can also oxidise low and high density lipoproteins and form conjugated dienes, thiobarbituric acid reacting substances (TBARS) and F2-isoprostanes (Camejo et al., 1998) (see section 1.2.7). Interestingly, haemin also causes covalent cross-linking of apoliprotein B with the formation of bityrosines and simultaneous formation of protein aggregates. This is pathophysiologically important, as low density lipoprotein aggregation relates to conversion of macrophages into foam cells, and therefore, haemin could be considered as an endogenous trigger of atherosclerosis (Miller and Shaklai, 1994)

1.2.7 Haem and LDL oxidation

Haem can also cause indirect vascular endothelial damage through its ability to mediate oxidative modification of low-density lipoprotein (LDL). Haem-mediated LDL oxidation involves coupled oxidative interactions between LDL, haem, oxidants and anti-oxidants. The first step of this process entails the spontaneous insertion of haem into LDL particles, thus promoting extensive oxidative modification of LDL. These modifications can be amplified by small amounts of hydrogen peroxide, PMN derived oxidants or preformed lipid hydroperoxides within the LDL. Depletion of α-tocopherol (an anti-oxidant) in LDL results in the formation of conjugated dienes, lipid hydroperoxides and TBARS. Haem can oxidatively modify both the lipid moiety of LDL as well as its apoprotein. The reactions between haem, LDL and peroxides result in the degradation of the haem ring and concomitant release of free iron, which exacerbates LDL oxidation. The release of free iron stimulates further oxidation of haem, fatty acids, cholesterol and apolipoprotein B100 in LDL particles. Accordingly, the iron chelator, desferrioxamine has been shown to attenuate

both oxidative modification of LDL and degradation of haem. Haemopexin also inhibits oxidative modification of LDL. *In vivo* studies have shown that activated PMNs potentiate oxidation of LDL catalysed by haem iron, and LDL oxidized by haem is extremely cytotoxic to endothelial cells. It is also hypothesized that free haemoglobin could damage vascular endothelial cells through haem-mediated oxidative modification of LDL. Specifically oxyhaemoglobin may be converted to methaemoglobin via inflammatory cell derived oxidants; as a consequence release of haem from methaemoglobin may be important in the oxidative modification of LDL (Balla et al., 2005).

1.2.8 Haem and inflammation

Inflammation is a defence mechanism utilised by organisms to protect them from pathogenic invaders, remove the damaged cells after injury and prevent further damage. The inflammatory response involves several stages: (a) the dilation of capillaries to increase blood flow; (b) microvascular structural changes and escape of plasma proteins from the bloodstream; (c) leukocyte adhesion; (d) elimination of pathogens; and (e) resolution of inflammation. Haem-mediated oxidation insults and inflammation are likely important in a wide variety of pathophysiological processes (Balla et al., 2000), such as renal failure, arteriosclerosis and heart transplant failure. Exposure of endothelial cells to haem *in vitro* has been shown to stimulate the expression of intracellular adhesion molecules (ICAM), vascular cell adhesion molecule1 (VCAM1) and endothelial leukocyte adhesion molecules (E-selectin), most probably through haem-mediated generation of intracellular reactive oxygen species and subsequent activation of transcription factors NF-KB, AP-1 and SP-1 signalling pathways (Lavrovsky et al., 1994; Shono et al., 1996). These activated

endothelial adhesion molecules will subsequently recruit leukocytes, resulting in one of the main characteristics of inflammation. Vascular haem can also cause endothelial cell injury, leading to inflammatory lesions and the formation of vascular inflammatory disorders (Wagener et al., 2001). Therefore, free haem can be termed a pro-inflammatory molecule that activates neutrophil responses. However, haem also up-regulates HO-1 which provides anti-inflammatory actions. Specifically, a small concentration of haem can have anti-inflammatory and cytoprotective properties through up-regulation of HO-1 and by stimulating the formation of the HO-1 products CO and BV (Hayashi et al., 1999). In contrast, a large amount of haem has deleterious effects on tissue via its pro-oxidative and pro-inflammatory function (Nath et al., 2001b), which cannot be sufficiently counter-balanced by the anti-oxidative and anti-inflammatory properties of HO-1. Increased levels of HO-1 expression have been clinically demonstrated in a wide variety of inflammatory conditions, such as ischaemia/reperfusion injury, atherosclerosis, asthma, Alzheimers disease and acute renal failure (Wagener et al., 2003). The production of HO-1 is greatly elevated in inflammatory cells during the resolution phase of inflammation (Willis et al., 1996), when the cytoprotective effect of HO-1 is mediated by down-modulation of adhesion molecules expression (Wagener et al., 2003).

1.3 Haemoglobins

1.3.1 Chemical structure and properties

Haemoglobin is a highly conserved molecule found in species ranging from single cell organisms to mammals; however, the role of haemoglobin varies in different organisms. For example, in bacteria haemoglobin can oxidise

NO to nitrate (Goretski and Hollocher, 1988), while in nematodes haemoglobin functions to eliminate oxygen and is thus critical for anaerobe survival (Minning et al., 1999). In mammals, haemoglobin is found in red blood cells and has a primarily respiratory function by carrying oxygen in blood and transporting carbon dioxide and hydrogen ion (Wells, 1999). HbA is the principal haemoglobin in human adults, consisting of two alpha chains and two beta chains combined in a tetrameric molecule (Figure 1-4). Each chain is folded into characteristic seven or eight a-helices (labelled A-H) which are linked by non-helical segments (e.g. EF, FG) and an N-terminal and C-terminal extension termed NA and HC respectively. The "haem pocket" is a crevice in each chain between the E and F helices, containing an oxygen binding haem group. Adults also have a minor haemoglobin (around 2% of total haemoglobin) called HbA_2 which has delta chains instead of beta chains found in HbA. Foetuses have their own distinct haemoglobin. Shortly after conception foetuses synthesize zeta chains (which are similar to alpha chains) and epsilon chains (similar to beta chains). During foetal life, the zeta chain is replaced by the alpha chain, and the epsilon chain is replaced by the gamma chain, which gives rise to HbF ($\alpha_2\gamma_2$) the major haemoglobin in the latter two-thirds of foetal life. After birth, the gamma chain is substituted with the beta chain to produce HbA. Haemoglobin synthesis requires coordinated production of haem and globin, in which haem is the prosthetic group that mediates reversible binding of oxygen by haemoglobin whilst globin is the protein that surrounds and protects the haem molecule. The complex biophysical characteristics of the complete haemoglobin tetramer allows fine control of oxygen uptake in the lungs and release in the tissues that is necessary to sustain life (Weber and Fago, 2004).

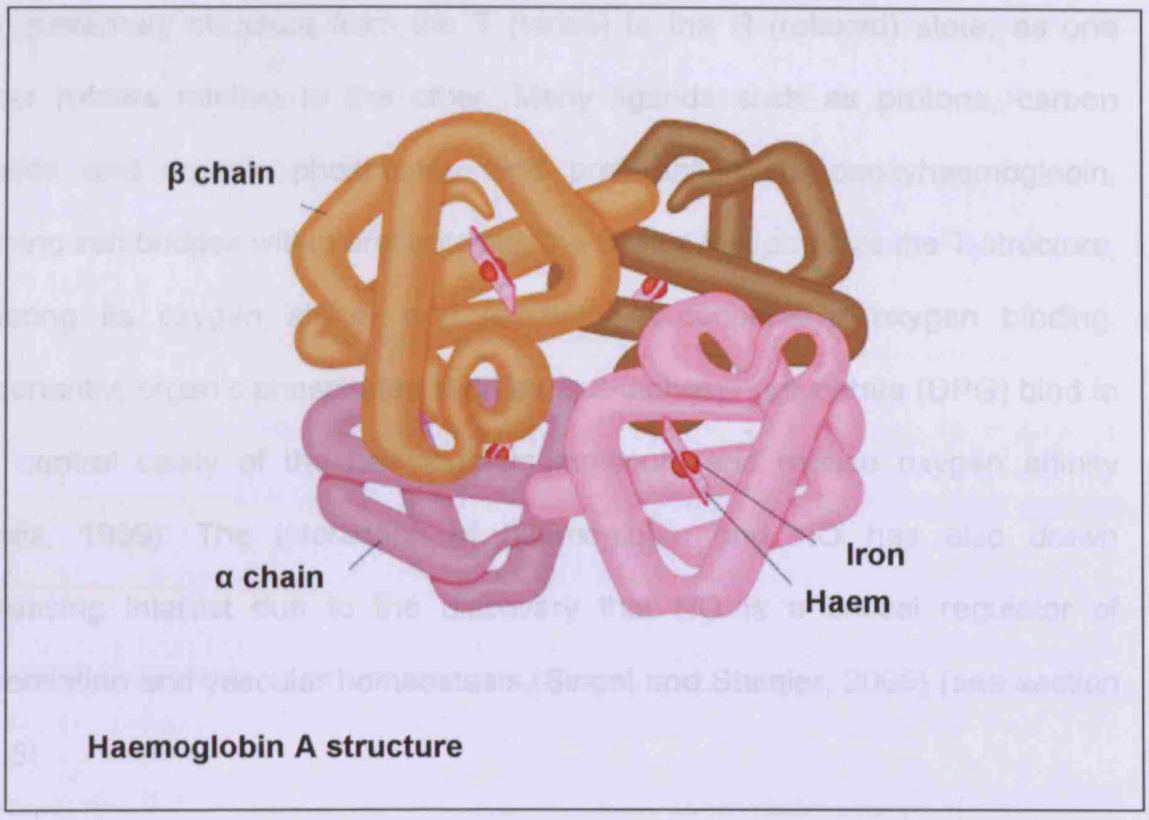


Figure 1-4. Structure of adult mammalian haemoglobin

1.3.2 Physiological role of haemoglobins

The physiological role of haemoglobins are a result of their intrinsic oxygen-binding properties and the effects of local environmental factors (e.g. pH, temperature and cofactor concentrations) that modulate oxygen binding. The functional adaptations are dependent on homotropic interactions i.e. cooperativity between oxygen binding to haem groups, and the generally inhibitory heterotropic ones between effector binding sites and the haems (e.g. decreased oxygen affinity at low pH, the Bohr effect). The homotropic and heterotropic allosteric interactions increase the oxygen capacitance (turnover) for oxygen tension differences between sites of oxygen loading (lungs) and unloading (tissues). Oxygen binding is associated with small changes in the tertiary structure of the segments near the haem moieties and a large shift in

the quaternary structure from the T (tense) to the R (relaxed) state, as one dimer rotates relative to the other. Many ligands such as protons, carbon dioxide and organic phosphates bind preferentially to deoxyhaemoglobin, forming salt bridges within and between the chains that stabilize the T-structure, lowering its oxygen affinity and resulting in cooperative oxygen binding. Importantly, organic phosphates such as 2, 3-diphosphoglycerate (DPG) bind to the central cavity of the haemoglobin molecule and reduce oxygen affinity (Wells, 1999). The interaction of haemoglobin and NO has also drawn increasing interest due to the discovery that NO is a critical regulator of vasodilation and vascular homeostasis (Singel and Stamler, 2005) (see section 1.6.5).

1.3.3 Haemoglobin gene regulation

The genes that encode the alpha globin chains are on chromosome 16 and those that encode the non-alpha globin chains are found on chromosome 11. There are multiple individual genes expressed at each site. Pseudogenes are also present at each location. The alpha complex is called the "alpha globin locus", while the non-alpha complex is called the "ß globin locus". The expression of the alpha and non- alpha genes is closely tuned as balanced gene expression is required for normal red cell function. Regulation of haemoglobin synthesis involves control of both porphyrin and polypeptide synthesis. The regulating factor is an accumulation of haem which, in the free form, is spontaneously oxidized to haemin. An accumulation of haemin diminishes the activity of ALA synthase, probably by repressing synthesis of the new enzyme, as well as directly inhibiting the existing enzyme, thereby reducing porphyrin synthesis. Haemin has this effect both in the primitive red blood cell

synthesizing haemoglobin and in other cells in which the cytochromes and other haem proteins are made. Haemin activates the synthesis of globin peptide by combining with an inhibitory protein. This mechanism keeps the synthesis of haem and globin in balance (Bunn, 1987).

1.3.4 Haemoglobinopathies

From time to time, mutations may occur in human HbA globin chains. If any change occurs close to the haem-oxygen binding site, near the central cavity or at some other site which destabilizes the structure of the molecule, it has the potential to interfere with the normal function of haemoglobin in oxygen transport. Nearly 1000 haemoglobinopathies have been described and they refer to a diverse group of inherited disorders characterized by reduced synthesis of one or more of the globin chains (thalassaemias) or the synthesis of structurally abnormal haemoglobin such as sickle cell disease (SCD). Furthermore, some structural haemoglobin variants are also inefficiently synthesized or the globin chain variants are so unstable that they are unable to form tetramers, resulting in a functional deficiency in the globin chain and a thalassaemic phenotype. Another group of haemoglobinopathies is referred to as hereditary persistence of foetal haemoglobin (HPFH), which is characterized by variable increases of HbF in otherwise normal adults (Clark and Thein, 2004).

As a group, the haemoglobinopathies are the commonest single gene disorder in the world and are found at high frequencies in tropical and subtropical regions where malaria is found to be endemic (Flint et al., 1998). There are four main categories of haemoglobin disorders that are associated with severe disease states and clinical significance (Clark and Thein, 2004):

- Sickle cell disease (major genotypes HbSS, HbSC, HbSβ$^+$Thal or HbSβ°Thal, and less common genotypes Hb SD ^{Punjab}, Hb SO^{Arab} and Hb S Lepore)
- ß-thalassaemia syndrome; including δß-thalassaemias and Hb E/ß-thalassaemias
- α-thalassaemia syndrome
- Haemoglobin variants resulting in haemolytic anaemias, polycythaemias and more rarely cyanosis.

Sickle cell disease is of major interest in the context of this thesis and will be described in detail in the following section.

1.4 Sickle cell disease (SCD)

SCD arises from a single point mutation in the beta-globin gene (glutamic acid is replaced by valine), which subsequently results in the production of the mutant haemoglobin HbS. There are a number of genotypes found in SCD, with the most prevalent being homozygous HbSS which accounts for two-thirds of all SCD cases. A smaller population of sickle patients carry the heterozygous genotypes, the most common being HbSC, HbSβ$^+$Thal and HbSβ°Thal, in order of frequency. The most severe disease is seen with the HbSS and HbSβ°Thal traits, followed by the milder HbSC genotype which in turn is more severe than HbSβ$^+$Thal. Significantly, there can be overlap of severity within these genotypes, so a patient with any one of these sickle traits can present with any clinical complications of SCD (see section 1.4.1) (Redding-Lallinger and Knoll, 2006).

The trigger for the diverse complications observed in SCD occurs at the molecular level. Specifically, the substitution of valine for glutamic acid in the

mutant HbS molecule triggers hydrophobic interactions with other haemoglobin molecules resulting in the formation of polymers. Deoxygenation causes HbS to polymerise (Figure 1-5), which then leads to erythrocyte rigidity, membrane damage, haemolysis and causes damage to tissues and organs (Bunn, 1997).

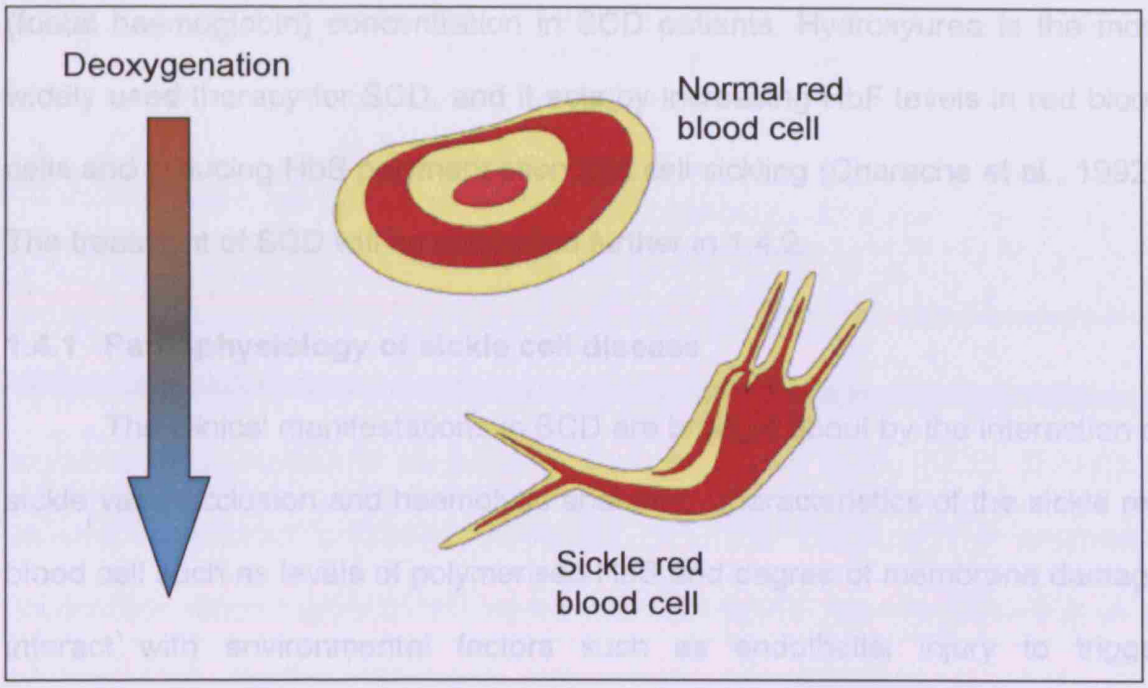


Figure 1-5. Deoxygenation triggers sickling of red blood cells

A fall in the partial pressure of oxygen triggers polymerisation of HbS molecules within the red blood cells which results in distortion of the red blood cell into the characteristic "sickle" shape.

Polymerisation of HbS is dependent on the cellular concentration of HbS. A classical feature of SCD is the presence of dense dehydrated erythrocytes, where the increased cellular haemoglobin concentration and an enhanced tendency to form polymers trigger the cell to assume the characteristic "sickle" shape. Cellular damage results in adhesive interaction between sickle cells, endothelial cells and leukocytes. The adherent cells may initiate vessel obstruction (vaso-occlusive crises), while the dense cells may become sequestered or destroyed in the microvasculature (Steinberg and Brugnara,

2003). The development of vaso-occlusive crises is seen as a hallmark of SCD. Irreversibly sickled cells lead to obstruction of blood vessel, resulting in tissue hypoxia and infarctions (Lawrenz, 1999).

The most clinically studied approach against "sickling" is increasing HbF (foetal haemoglobin) concentration in SCD patients. Hydroxyurea is the most widely used therapy for SCD, and it acts by increasing HbF levels in red blood cells and reducing HbS polymerisation and cell sickling (Charache et al., 1992). The treatment of SCD will be discussed further in 1.4.2.

1.4.1 Pathophysiology of sickle cell disease

The clinical manifestations in SCD are brought about by the interaction of sickle vaso-occlusion and haemolytic anaemia. Characteristics of the sickle red blood cell such as levels of polymerised HbS and degree of membrane damage interact with environmental factors such as endothelial injury to trigger haemolysis and vaso-occlusion. Many factors can provoke endothelial activation and damage such as inflammation and circulatory shear stresses. Damage to the endothelial cell facilitates adhesive interactions between sickle red blood cell, endothelial cells and leukocytes which are mediated by several adhesion molecules and their ligands. These adhesive interactions between cells can result in delay of red blood cell passage through the vasculature, which could enable HbS polymerisation, cell deformity and vaso-occlusion to occur (Steinberg and Brugnara, 2003). Furthermore, reticulocytes have adhesive ligands expressed that facilitate endothelial interactions. Another common finding in SCD is an abundance of activated neutrophils which are also more adherent to endothelial cells (Jison et al., 2004).

Elevated levels of plasma haemoglobin found in SCD are due to premature destruction of red blood cells. The release of haemoglobin can lead to further haemolysis in SCD by interaction of haem with red cell membranes and subsequent oxidative damage (Kumar and Bandyopadhyay, 2005). Indeed, the amount of incorporated haem is found to be higher in pathological cells of SCD than normal circulating cells, and may contribute to the removal of aged red blood cells from the circulation through haemolysis.

Another consequence of elevated free haemoglobin levels in sickle blood is the interaction with NO. Plasma haemoglobin converts NO to biologically inert nitrate, and lysed sickle red blood cells liberate arginase which destroys L-arginine, the substrate for the production of NO (Reiter et al., 2002). Therefore, an overall reduced concentration of NO in sickle blood and a decreased endothelial bioavailability of NO impairs its homeostatic vascular functions (e.g., inhibition of platelet activation, repression of cell-adhesion molecules), consequently promoting vasoconstriction which increases the possibility of vaso-occlusion (Steinberg and Brugnara, 2003).

The reduction in NO bioavailability has a key role in the development of stroke, pulmonary hypertension, leg ulcers and priapism in SCD. In addition, an increase in the blood viscosity of sickle patients increasing the incidence of vaso-occlusion and gives rise to clinical phenotypes such as osteonecrosis, acute chest syndrome and painful episodes (Steinberg, 2006). In fact, the most prevalent consequence of red cell sickling at the microscopic level is the development of sickle crisis, the characteristic symptom associated with the disease. These can be divided into vaso-occlusive (painful) crises, aplastic or megaloblastic crisis, sequestration crisis and haemolytic crisis. Within the sickle

population, the severity and frequency of crisis varies due to inherited or acquired factors, such as sickle genotype and concomitant infection (Beutler, 2001; Di Nuzzo and Fonseca, 2004).

Vaso-occlusive crises, a consequence of sickle cell-mediated vessel obstruction resulting in tissue hypoxia and infarction, are seen as the hallmark of the disease and are associated with fever and pain at the site of tissue infarction (Steinberg and Brugnara, 2003). SCD patients present most commonly with pain in their bones, chest or abdomen (due to splenic crisis), and the average crisis rate is less than 1 (0.8) a year (Marchant and Walker, 2003). In sickle patients with higher rates of crisis, there is an associated increase in morbidity and mortality. The most serious complication of SCD is infarction of the cerebral vessels resulting in stroke and is most commonly associated with childhood (Lawrenz, 1999).

1.4.2 Treatment of sickle cell disease

An acute presentation of sickle crisis is managed palliatively, with appropriate pain relief and blood transfusion if necessary; however, the strategy for long-term SCD management aims to prevent sickling of red blood cells and thereby reduce the consequent cellular and tissue damage. Polymerization of HbS is the most important factor in sickling and a rise in HbF decreases intracellular polymerization of HbS. Therefore, the search for an anti-sickling agent has focused on compounds which stimulate HbF synthesis. The drug hydroxyurea, which is the standard treatment in SCD, is a ribonucleotide reductase inhibitor that after absorption is reported to liberate NO (King, 2004). Hydroxyurea elicits clinical benefit by decreasing the terminal differentiation of erythroid stem cells, which leads to myelosuppresive-induced HbF synthesis

(Charache et al., 1992). SCD patients on hydroxyurea therapy have shown significant decrease in the frequency of crisis, acute chest syndrome, transfusion requirements and hospitalisations (Charache et al., 1995). Furthermore, combination therapy with hydroxyurea and erythropoietin has been shown to further increase HbF production in sickle patients (Little et al., 2006). Erythropoietin treatment has also been used on its own in SCD to improve the severe anaemia prevalent in this disease (Steinberg, 1991).

Transfusion therapy is also widely used in the management of SCD, usually administered as packed red blood cells. The key goals of red blood cell transfusion in SCD include: increasing total haemoglobin levels to improve the oxygen transport capacity of blood; reducing blood viscosity and increasing oxygen saturation through dilution of the HbS-containing red blood cell population; and suppressing endogenous production of sickle red blood cells by increasing tissue oxygenation. The benefits of transfusion therapy in sickle patients include improvement in anaemia, prevention and resolution of vaso-occlusive crisis that result in acute chest syndrome, stroke and other ischaemic organ damage in SCD (Josephson et al., 2007).

Recently, NO therapy has also been suggested as a treatment for complications of SCD. Inhaled NO therapy has had beneficial effects in patients presenting with acute chest syndrome (Vichinsky et al., 1997) and has reduced pain scores and opioid use in children with acute crisis (Weiner et al., 2003). Increasing endogenous NO production with administration of the NO substrate arginine has also shown promising effects in pulmonary complications (Morris et al., 2003). However, further clinical trials are required to elucidate the benefit of NO administration in SCD.

Bone marrow transplantation in SCD would eliminate the source of the defective red blood cells and is the only curative therapy available. This procedure, however, has not been widely utilized due to its high mortality rate. Fortunately, recent advances in immunosuppressive therapy and supportive care has reignited interest in this therapeutic approach for SCD and encouraging results from clinical trials with children show a 90% long-term survival rate (Vermylen et al., 1993). However, drawbacks of this approach include the difficulty in finding matched donors and limitation of the procedure to childhood due to the increased mortality associated with transplant in adults.

1.4.3 Haem oxygenase and sickle cell disease

Chronic haemolysis in SCD results in up-regulation of haem catabolising enzyme systems to eliminate excess haemoglobin released. Indeed, HO-1 induction has been demonstrated in various cell types in SCD, including leukocytes (Jison et al., 2004), kidney tissue and circulating endothelial cells (Nath et al., 2001a). In leukocytes from sickle patients an increase in HO-1 protein and mRNA levels were observed concomitant with increase in plasma carboxyhaemoglobin and BR, suggestive of a concurrent increase in haem oxygenase activity (Jison et al., 2004). A widespread induction of HO-1 has also been found in the kidney of sickle patients, where raised levels of the enzyme were detected in renal tubules, interstitial cells and smooth muscle cells of the renal vasculature (Nath et al., 2001a). Furthermore, these patients exhibited increased HO-1 expression in circulating endothelial cells separated from blood.

The raised levels of HO-1 in circulating leukocytes and endothelial cells could act as a dynamic compensatory response to the repetitive ischaemia-reperfusion injury that is characteristic of SCD, whereas induction of HO-1 in

organs, such as the kidney, could also protect tissue against the deleterious effects of vascular injury. For instance, the induction of endothelial HO-1 has been well established as an adaptive mechanism to protect the endothelium against the damaging effects of the pro-oxidants haem and haemoglobin (Balla et al., 2003), and prevent endothelial cell activation and damage which are important contributors to SCD pathology. In addition, the vasodilatory properties of HO-1 generated CO could hinder the development of vaso-occlusive events, reducing the incidence of tissue infarction. Furthermore, the potent anti-inflammatory properties of HO-1 may also contribute to protect against cellular and tissue damage in SCD. For example, increased HO-1 expression has been shown to be associated with inhibition of the expression of adhesion molecules associated with endothelial cell activation (Soares et al., 2004), which could reduce the incidence of red blood cell and endothelial cell interaction in SCD, thereby decreasing the frequency of vessel obstruction and resultant tissue damage.

Intriguingly, the biproducts of HO-1 induction have also been shown to have an action in SCD. A challenging therapeutic trial by Beutler, undertaken over 30 years ago, showed that CO gas administration could significantly prolong red cell survival in SCD (Beutler, 1975). In that study, sickle patients inhaled low levels of CO (1000-2000 ppm) intermittently (every 3-4 h) over 5 day courses. Blood carboxyhaemoglobin levels reached levels up to 15% and no toxic effects were reported by the patients. Analysis of sickle blood after CO administration demonstrated a decreased rate of haemolysis and an increase in haemoglobin concentration, resulting in an overall increase in haematocrit. The increase in sickle red blood cell lifespan was attributed to the CO binding to the

HbS molecule, which favoured the more stable oxyhaemoglobin conformation of the molecule and therefore reduced the likelihood of sickling and haemolysis. Furthermore, CO treatment also increased haemoglobin concentration by stimulating erythropoiesis. Therefore, CO treatment showed intriguing anti-sickling properties as well increasing the oxygen-delivery capacity of sickle blood by increasing the red blood cell mass which could result in reduction of sickle crisis. These beneficial effects of CO were observed after continued administration of CO gas over a period of days. However, results from acute administration of CO for sickle pain relief were inconclusive, with only some patients reporting pain relief. The potential toxic effects of CO hindered continued investigation into the possible therapeutic effects of CO in SCD. However, mounting evidence pointing towards the potent protective properties of CO suggests that the therapeutic role of CO in SCD requires further exploration.

It has also been demonstrated that BR plays an important anti-oxidant role in SCD (Dailly et al., 1998). The anti-oxidant activity of BR was investigated in plasma from sickle patients and BR levels were shown to be the limiting factor in sickle plasma oxidant activity. The investigators propose that in SCD the increase in plasma BR levels caused by haemolysis and oxidative stress could confer protection on sickle erythrocyte membranes from peroxidation and thereby reduce the incidence of haemolysis. Intriguingly, high BR levels may also contribute to the surprising lack of atherosclerotic coronary heart disease in the sickle patient population (Barrett, Jr. et al., 1984). Therefore, the potent anti-oxidant properties of HO-1 generated BR could counteract the pro-oxidant environment generated by haemolysis.

The treatment of SCD with NO offers another potential link between HO-1 and SCD. It has been established that hydroxyurea exerts its effects via the release of NO (Gladwin et al., 2002) and inhaled NO therapy has itself shown beneficial effects in SCD (Reiter and Gladwin, 2003). As NO itself is a potent inducer of HO-1, it is possible that some of the beneficial effects of hydroxyurea in SCD may be mediated through the induction of HO-1. Indeed, increasing evidence suggests that the mechanism of clinical benefit of hydroxyurea may include HbF-independent components. For example, other possible mechanisms of action for hydroxyurea in SCD include effects on adhesion-molecule expression, vascular reactivity, red-cell deformability or erythropoietin production (Steinberg, 2006). It is possible that HO-1 induction directly or indirectly mediates some of these effects of hydroxyurea; for example, erythropoietin treatment has been demonstrated to raise HO-1 levels in patients with kidney dysfunction (Calo et al., 2003).

Collectively, these studies taken together suggest there may be a potential role for HO-1 in the treatment of SCD. The induction of HO-1 in SCD could be a physiological response to counterbalance the oxidative damage generated by haemolysis, and harnessing the protective properties of HO-1 by enhanced induction or administration of HO-1 biproducts CO and BR may present new therapeutic avenues for the treatment of SCD. Accordingly, Belcher and colleagues have recently shown that inducing HO-1 expression in transgenic sickle mice inhibited the hypoxia/reoxygenation induced stasis found in this model of SCD (Belcher et al., 2006). Furthermore, they reported reduced leukocyte-endothelium interactions as well as NF-κB, VCAM-1 and ICAM

expression, suggesting that HO-1 plays a vital role in the inhibition of vaso-occlusive crises in transgenic sickle mice.

1.5 Hypoxia

Tissue hypoxia is a state of oxygen deficiency and may occur in biological systems as a consequence of reduction in partial pressure of oxygen, restricted intake and inadequate transport of oxygen or the inability of tissue to use oxygen. Hypoxia characterizes various physiological states such as arteriosclerosis, fibrosis and neoplasia. Mammalian organisms have evolved specific mechanisms to counteract the effect of reduced oxygen levels resulting in tissue adaptation to a low oxygen environment. A key physiological response to hypoxia is stimulation of the kidney to synthesise and secrete increased amounts of the hormone erythropoietin, which stimulates the production of red blood cells and therefore enhances the oxygen carrying capacity of the blood and improving oxygen delivery to hypoxic tissues. Other physiological responses to hypoxia include enhanced expression of promoters of angiogenesis and glycolytic enzymes for alternative ATP generation pathways (Bunn and Poyton, 1996). Central to the expression of these hypoxic-sensitive genes is the redox modification and phosphorylation of specific transcription factors such as hypoxia-inducible factor-1 (HIF-1) (Bunn and Poyton, 1996). A hypoxic environment also induces the expression of the physiologically important haem oxygenase and nitric oxide synthase enzymatic pathways which generate essential signalling molecules (Maines, 1997) and this subject will be explored further in section 1.5.3.

1.5.1 Hypoxia inducible factor-1

Hypoxia is able to regulate the expression of several target genes through the actions of the vital transcription factor HIF-1 which is expressed widely in mammalian cells, including endothelium and vascular smooth muscle (Schmedtje, Jr. et al., 1996). HIF-1 is a heterodimeric complex of basic-helix-loop-helix proteins comprising of two distinct subunits, HIF-1α and HIF-1β. Regulation of the HIF-1α subunit determines the biological activity of HIF-1 which occurs at the protein level. Under normoxic conditions, an oxygen dependent HIF-specific prolyl hydroxylase enzyme targets the HIF-1α subunit and hydroxylates two proline residues which allows binding of the Von Hippel-Lindau tumour suppression protein, priming the HIF-1α subunit for proteosomal degradation. However, hypoxia diminishes the activity of the HIF hydroxylase which allows HIF-1α to escape proteolysis and allows the HIF-1 complex to activate the transcription of hypoxia–inducible genes (Ratcliffe, 2006). The transcriptional targets for HIF-1 play central roles in enhanced angiogenesis and erythropoiesis as well as vasomotor regulation, matrix metabolism, cell proliferation, energy metabolism and many other cellular and systemic responses to hypoxia (Semenza, 2000).

1.5.2 Erythropoietin

In response to hypoxia the kidney increases erythropoietin production and stimulates erythropoiesis, increasing red cell production and hence the oxygen carrying capacity of the blood. Therapeutically recombinant human erythropoietin has been used to improve haematocrit and lower blood transfusion requirement. However, the action of erythropoietin is not restricted to the erythroid compartment and erythropoietin-receptors are present on

several non-erythroid cell types and are involved in the induction of a range of cellular responses, including angiogenesis, chemotaxis and inhibition of apoptosis (Lappin et al., 2002).

Intriguingly, the anti-apoptotic action of erythropoietin has been postulated to act through HO-1 (Calo et al., 2006) via erythropoietin's stimulatory effect on protein kinase B activity; this enzyme in turn phosphorylates HO-1 resulting in an increase in the enzyme's activity. Furthermore, increased expression of monocyte HO-1 has been observed in haemodialysis patients on erythropoietin treatment (Calo et al., 2003), suggesting a direct effect of erythropoietin on HO-1 levels. It is possible that induction of HO-1 by hypoxia-dependent erythropoietin could be an adaptive mechanism to counteract hypoxic tissue injury mediated through the potent protective effects of haem degradation products CO and BV. The hypoxic induction of HO-1 has in fact been demonstrated in various cell types and will be discussed further in the following section.

1.5.3 Hypoxia and haem oxygenase

The cytoprotective stress protein HO-1 is emerging as a major hypoxia-inducible protein in mammalian cells. Hypoxia-mediated HO-1 induction has been observed in both rat pulmonary endothelial and aortic smooth muscle cells (Lee et al., 1997), with HIF-1 determining the HO-1 response in smooth muscle cells and another transcription factor AP-1 responsible for endothelial induction (Hartsfield et al., 1999), suggesting that HIF-1 may not mediate hypoxic induction of HO-1 in all cell types. Indeed, bovine aortic endothelial cells exposed to hypoxia elicited both enhanced HO-1 and iNOS expression (Motterlini et al., 2000) suggesting an alternative hypoxic signal transduction

mechanism for endothelial HO-1 induction; that is, hypoxia-mediated induction of iNOS can stimulate HO-1 protein production through the generation of NO. Furthermore, a decrease in the ratio of reduced glutathione/oxidised glutathione was observed in bovine aortic endothelial cells after induction of HO-1 by hypoxia which was postulated to be a consequence of an interaction between reduced glutathione and NO leading to the formation of S-nitrosothiols. These data, along with mounting evidence of hypoxia-mediated HO-1 induction in cells of vascular origin (Ryter and Choi, 2002), suggest that hypoxia-induced HO-1 may represent an adaptive response to vascular oxidative injury. Hypoxic induction of both HO-1 and NOS systems would increase the availability of the second messenger molecules NO and CO, which are required to regulate vascular functions such as vasodilation, inhibition of platelet aggregation and expression of vasoconstrictors as well as smooth muscle proliferation.

1.5.4 Hypoxia and sickle cell disease

The role of hypoxia in SCD has many facets, ranging from the effects on the molecular configuration of HbS to the development of clinical phenotypes. A fall in the oxygen level is the trigger for the polymerisation of the mutant HbS molecule within the red blood cell (Bunn, 1997). Accumulation of polymers will eventually distort and "sickle" the red blood cell, which due to its lack of deformability can lodge in end-arterioles and capillaries resulting in vaso-occlusive crises and tissue infarction. Regional hypoxia caused by obstruction of vasculature by damaged red blood cells, will generate more sickled cells and increase the likelihood of haemolysis and subsequent free haemoglobin release will activate the endothelium to express adhesion molecules precipitating more vaso-occlusive events (Bunn, 1997). Therefore, tissue hypoxia is prevalent in

SCD patients (Vichinsky et al., 1997) and occurs especially in venular beds where the sluggish flow favours the deoxygenation and polymerisation of HbS. Hence, hypoxia can be seen as a central factor in the pathophysiology of SCD.

The physiological response to hypoxia is also altered in SCD, where the hypoxia driven erythropoietin response is found to be diminished in patients (Sherwood et al., 1986). As red blood cells containing polymerised HbS have a lower affinity for oxygen, their release of oxygen to tissues is enhanced compared to cells containing the normal HbA, thereby blunting the normal erythropoietin response to hypoxia. Conversely, patients on hydroxyurea therapy with elevated levels of HbF display higher elevated erythropoietin levels than subjects not receiving hydroxyurea as HbF has increased affinity for oxygen and releases less oxygen at a given oxygen tension (Charache et al., 1992). Furthermore, the prevalence of kidney disease in the SCD population may further diminish the erythropoietin response to hypoxia by impairing hormones production. Therefore, erythropoietin has been utilised as a therapeutic tool in SCD to raise haematocrit and also has generated interest as a pharmacological agent to augment HbF production with or without concomitant hydroxyurea therapy (Little et al., 2006).

Respiratory complications, such as acute chest syndrome and pulmonary hypertension, are one of the most common causes of hospitalisation for SCD patients; they are a direct consequence of haemolysis and subsequent NO scavenging and can act as another contributor to hypoxia in this disease (Vichinsky et al., 1997). Development of respiratory complications in SCD causes hypoventilation and hypoxaemia, thus promoting further intra-pulmonary sickling of red blood cells and resulting in inflammatory damage to the

pulmonary endothelium (Vichinsky and Styles, 1996). However, inhaled NO therapy has been successful in ameliorating the effects of pulmonary hypertension (Sullivan et al., 1999).

The role of hypoxia in SCD pathophysiology is of paramount importance and as low oxygen levels have been shown to induce erythropoietin, NO and HO-1, it is tempting to suggest that there may be an inter-relationship between these three components. Indeed, the mechanism of action of erythropoietin or NO-based therapy in SCD could likely involve HO-1.

1.6 Nitric oxide

1.6.1 Chemical properties and reactions of NO

NO is an uncharged free radical that is involved in a wide array of physiological processes including smooth muscle relaxation, neurotransmission, platelet aggregation and host defense mechanisms (Nathan, 1992). NO is also thought to have a role in the pathology of several inflammatory disease states such as arthritis, myocarditis, colitis and nephritis. Furthermore, NO has also been implicated in other pathological conditions such as cancer, diabetes and neurodegenerative diseases (Davis et al., 2001). The downstream effects of NO depend on its local concentrations and availability of target molecules. For example, at low concentrations NO can activate cGMP-dependent pathways; cGMP in turn can regulate various physiological events such as vasodilatation and neurotransmission (Krumenacker, 2004). At higher concentrations, NO rapidly reacts with superoxide anion to form peroxynitrite, which results in nitration of proteins (specifically at tyrosine residues) which in turn are involved in diverse cellular physiological processes (Bian et al., 2003). Another

mechanism by which NO can elicit biological effects is by S-nitrosylation, which is the covalent attachment of the NO molecule to sulphydryl residues of proteins. The resulting formation of S-nitrosothiols is a post-translational protein modification that is widely involved in redox-based cellular signalling (Sun et al., 2006). Interestingly, NO can modulate calcium-activated potassium channel activity by either direct S-nitrosylation of the channel protein, by activation of the cGMP pathway or both (Weiger et al., 2002). The diverse mechanisms of action of NO and its numerous biological effects underlie the physiological importance of this molecule.

1.6.2 NO synthesis

The enzyme that produces NO is NO synthase (NOS) of which there are at least 3 isoforms. Each isoform comes from a distinct gene product. NOS-1, also called neuronal NOS (nNOS), is found in high levels in neuronal cells as well as in some non-neuronal tissue. Neuronal NOS is activated during conditions of elevated calcium by calmodulin, and is thought to produce NO as a neurotransmitter (Bredt and Snyder, 1992). NOS-2, also known as macrophage or inducible NOS (iNOS), can be induced in many cell types such as macrophages, hepatocytes and smooth muscle cells. Inducible NOS is not generally present in resting cells but is produced in response to lipopolysaccharide (LPS) and cytokine stimulation as a cytotoxic response mechanism (Nathan, 1992). The NOS-2 isoform is the only one that is activated independent of calcium, which therefore requires the enzyme to be permanently bound to calcium/calmodulin once synthesized. NOS-3, also known as endothelial (eNOS), was the enzyme originally discovered to produce the endothelium-derived relaxing factor which was later identified to be NO (Nathan,

1992) (Figure 1-6). NOS-3 is also activated by raised levels of intracellular calcium and calmodulin binding. NOS-1 and NOS-3 are commonly referred to as constitutive NOS enzymes due to their constitutive expression and regulation by calcium/calmodulin. L-arginine is the substrate used by the NOS enzymes to produce NO. NOS enzymic activity also requires molecular oxygen, NADPH, tetrahydrobiopterin, flavin mononucleotide, flavin adenine dinucleotide and haem (Figure 1-6). The reaction results in the production of NO and L-citrulline (Marletta, 1994).

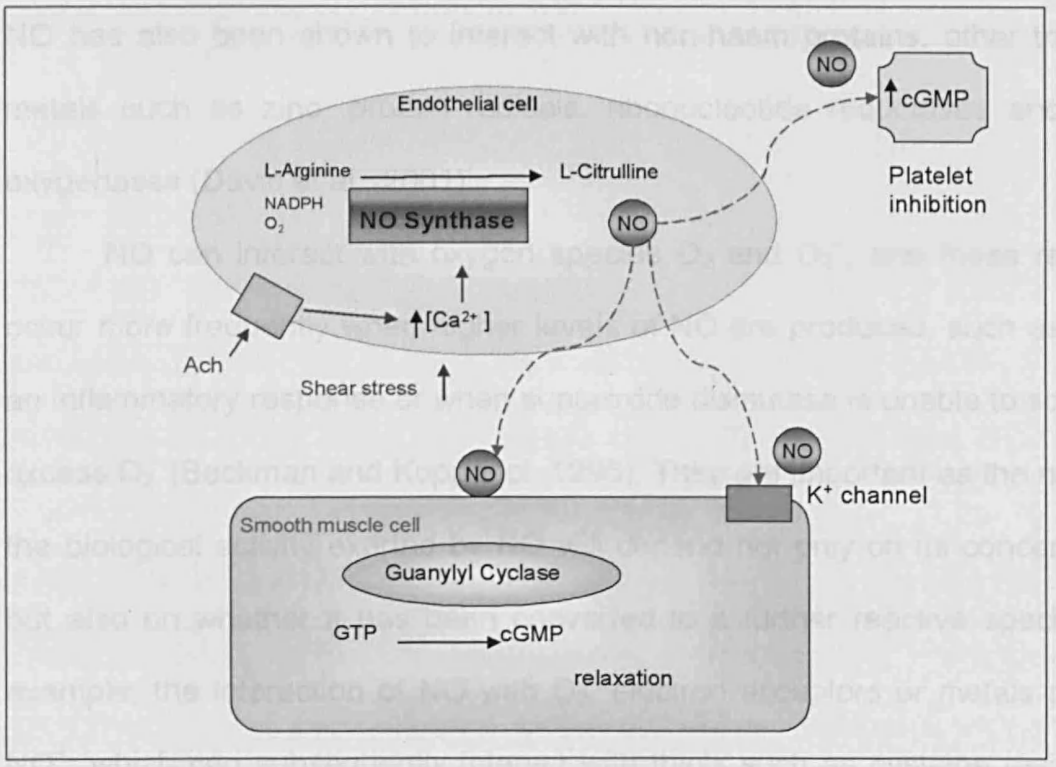


Figure 1-6. NO synthesis and action

NO is produced from L-arginine through the action of NO synthase (which can be stimulated by various mechanisms including through acetylcholine (Ach) receptors or shear stress) in the endothelial cell. It has numerous physiological functions including platelet inhibition and regulating vascular tone through smooth muscle relaxation.

1.6.3 Interaction of NO with biological molecules

NO contains an unpaired electron, making it a highly reactive and unstable free radical and resulting in the molecule reacting with other substances that have unpaired electrons. For example, its interaction with metal complexes, oxygen (O_2) or superoxide anion ($O_2^{\cdot-}$) results in multiple effects and downstream events depending on the localization and amount of NO and its substrates. NO concentrations are relatively low (<1 μM) under normal biological conditions and the gas will most likely react with haem complexes in proteins including sGC, haemoglobin, cytochrome p450 or protoporphyrin IX. NO has also been shown to interact with non-haem proteins, other transition metals such as zinc, protein radicals, ribonucleotide reductases and cyclo-oxygenases (Davis et al., 2001).

NO can interact with oxygen species O_2 and $O_2^{\cdot-}$, and these reactions occur more frequently when higher levels of NO are produced, such as during an inflammatory response or when superoxide dismutase is unable to scavenge excess $O_2^{\cdot-}$ (Beckman and Koppenol, 1996). They are important as the nature of the biological activity exerted by NO will depend not only on its concentration, but also on whether it has been converted to a further reactive species. For example, the interaction of NO with O_2, electron acceptors or metals produce NO^+, which can subsequently interact with thiols such as cysteine residues of proteins (Gaston, 1999). This can then result in S-nitrosylation of the thiol. Protein nitrosylation is a chemical reaction that has been shown to affect the function of numerous proteins, including transcription factors and signalling molecules; for example, DNA binding activities of NF-κB and c-junk are inhibited when they are nitrosylated and hence affect gene expression (Klatt et al., 1999).

It has been proposed that due to the very short half-life of NO in plasma, S-nitrosylation of plasma proteins such as albumin and haemoglobin may lead to stabilization and prolong the pharmacological action of NO (Stamler et al., 1992).

Under certain conditions, NO can also interact with $O_2^{-\cdot}$, resulting in the formation of the strong oxidant peroxynitrite ($ONOO^-$) (Huie and Padmaja, 1993). Peroxynitrite can lead to tyrosine nitration which is an addition of a NO_2 group to the phenol ring in the tyrosine. Nitration of a tyrosine in proteins can alter their function.

1.6.4 NO and haemoglobin binding

NO is a free radical with ligand properties similar to those of O_2 and CO which bind to oxyhaemoglobin. The affinity of NO for oxyhaemoglobin is much higher than that of O_2 or CO (Rifkind et al., 2004). One of the most important biological effects of NO involves the reaction with guanylate cyclase in smooth muscle cells, which produces vasodilatation. The endothelial NOS in the vascular endothelial cells provides the NO for this reaction. Some of the NO produced in the endothelial cells also diffuses into the lumen and may play a role in red blood cell function. In fact, a role for haemoglobin in the transport of NO to the vasculature has been proposed (Jia et al., 1996a) (see section 1.6.5).

Other NO species will also readily interact with haemoglobin. For example, peroxynitrite will directly react with thiol-containing compounds as well as haem proteins. The reaction of peroxynitrite with oxyhaemoglobin produces Fe(IV)ferrylhaemoglobin. Peroxynitrite can also react with methaemoglobin (Rifkind et al., 2004). An interesting reaction between nitrite and haemoglobin

has been postulated. Nitrite is present in plasma at a concentration of 150 nM - 1 μM and it reflects NO production from endothelial NO synthase enzymes (Gladwin et al., 2000). Nitrite can diffuse into the red blood cell and react with haemoglobin, oxidizing it (Kosaka et al., 1982). As the partial pressure of O_2 is lowered, the nitrite can react with deoxyhaemoglobin to regenerate NO and methaemoglobin (discussed further in section 1.6.5).

1.6.5 NO and haemoglobin interaction: physiological implications

NO produced by endothelial cells, apart from diffusing into surrounding smooth muscle cells, can also find its way into the flowing blood where it can interact with plasma constituents, especially oxyhaemoglobin. Under certain pathological conditions, such as haemorrhagic or subarachnoid shock, there is an accompanied over-production of NO, as well as raised levels of free haem/haemoglobin due to haemolysis. NO binds avidly to haemoglobin, and this interaction has led to a flurry of research and debate over the biological significance behind this reaction. It has been proposed that the binding of NO to haemoglobin may have an "oxygen-sensing" role, though the mechanism for this action has led to much discussion (Singel and Stamler, 2004). Stamler and co-workers have proposed that haemoglobin may bind NO at a specific cysteine residue to form S-nitroso-haemoglobin (SNO-haemoglobin) in its R (oxygenated) state. The NO could then be released from haemoglobin in its T (deoxygenated) state, resulting in NO-dependent vasodilatation in hypoxic conditions. Specifically, Stamler (Jia et al., 1996b) proposed a mechanism by which deoxyhaemoglobin could take up both O_2 and NO in the lung and then, upon transit to a site of low pO_2, liberate both gases. SNO-haemoglobin on its own was demonstrated to have potent vasodilatory activity and, therefore, a

possible role for haemoglobin in transporting NO was suggested. Most significantly, this activity is potentiated by the deoxygenation of haemoglobin. Therefore haemoglobin may sense ambient O_2 levels through its O_2 binding function and adjust NO bioavailability. In turn, red blood cells can cause microvessels to dilate or constrict, with SNO-haemoglobin as an intermediate. This model offers a mechanism for matching tissue perfusion with metabolic demands (Singel and Stamler, 2004).

A different mechanism has been proposed by Gladwin and colleagues, where the anion nitrite serves as a storage pool and can be converted to NO through haem-based reduction by deoxyhaemoglobin. Specifically, Gladwin proposed that nitrite could be converted into NO in the blood, and the released NO could induce local dilatation and increase regional blood flow. The essential feature of the nitrite haemoglobin hypothesis is that the interaction of nitrite with deoxyhaemoglobin generates a diffusible vasodilator with similar properties to NO. The barrier to diffusion that exists at the red cell membrane will limit the ability of red cells to destroy NO generated in the extracellular space and allow diffusion of red-cell generated NO to smooth muscle tissue (Gladwin et al., 2004). The major fundamental difference between the nitrite/haemoglobin hypothesis and the SNO-haemoglobin hypothesis is that haemoglobin does not carry the vasodilatory agent and that NO release is dependent upon the differential reactivity of nitrite with haemoglobin in the oxygenated and deoxygenated state, rather than oxygen-dependent conformational change that affects the reactivity of haemoglobin bound S-nitrosothiol (Gladwin and Schechter, 2004).

1.6.6 NO and haemoglobin interaction: pathophysiological implications

The high affinity of haemoglobin for NO poses significant problems when the endothelium is exposed to high concentrations of free haemoglobin, for example after haemolysis (Patel, 2000). Peripheral vasoconstriction (Sampei et al., 2005) has been largely attributed to scavenging of NO by haemoglobin, and excessive vasoconstriction can limit the use of cell-free haemoglobin as a blood substitute to carry O_2. Haemoglobin in the plasma is a more effective sink for NO than haemoglobin in red blood cells due to the diffusion limitation of NO in the red blood cell (Huang et al., 2001). Apart from causing vasoconstriction, the scavenging of NO by haemoglobin also has additional effects as NO serves many functions in maintaining a healthy vasculature. NO has been proposed to be an anti-atherogenic agent with mechanisms of action including stimulation of anti-oxidant enzyme synthesis, inhibition of platelet aggregation, down regulation of pro-inflammatory adhesion molecules, or direct inhibition of lipid peroxidation reactions (Hogg and Kalyanaraman, 1999). It is possible that the scavenging of NO will interfere with all these NO-dependent pathways. Under physiological conditions NO has been suggested to be protective against cell death. NO scavenging by cell-free haemoglobin may sensitise these cells making them more susceptible to cell death and contribute to haemoglobin-dependent toxicity. Indeed, haemoglobin promotes both necrotic and apoptotic death of endothelial cells, but whether this is NO-dependent is not known (Goldman et al., 1998). Scavenging of NO by haemoglobin is not always detrimental and has potential therapeutic benefits in the treatment of sepsis and conditions associated with hypertension such as stroke (Privalle et al., 2000).

1.6.7 Haem-nitrosyl complex formation

Due to the high affinity of NO for haem or haemoglobin, it is not surprising that the formation of nitrosyl-haemoglobin (HbNO) or haem-nitrosyl (H-NO) complexes may also occur when NO and haemoglobin are co-incubated. These complexes have been shown to form under physiological conditions. Indeed, ingestion of foods rich in nitrates, such as fresh fruit and vegetables, have been shown to increase HbNO levels in blood and have been suggested to have a role in the improvement of cardiovascular diseases (Tsuchiya et al., 2004). Circulating HbNO levels also increase in conditions such as endotoxic and haemorrhagic shock (Davies et al., 2005), which is not surprising as these conditions are characterized by increased levels of both haem (through haemolysis) and NO (endogenous production). In these situations, there is also a high level of intracellular H-NO complexes found in the liver, suggesting that H-NO may act as a substrate for HO-1 which is found in abundance in the liver (Davies et al., 2005).

1.6.8 NO interaction with haem oxygenase

Not only is NO a potent inducer of HO-1, but it can also affect the regulation of the enzyme. Indeed, excessive production of NO species resulting in nitrosative stress has been demonstrated to result in up-regulation of endothelial HO-1 (Foresti et al., 1999). There is a complex relationship between NO and HO-1, where NO can regulate HO-1 gene and protein synthesis as well as modulating the breakdown of haem by both HO-1 and HO-2. A direct reaction between NO and HO-1 breakdown products BR and BV has also been demonstrated (Kaur et al., 2003).

1.6.9 Up-regulation of HO-1 by NO

NO and NO-releasing agents differentiate themselves from other inducers of HO-1 because as well as producing oxidative stress they inflict an additional cellular threat, termed nitrosative stress (Foresti et al., 1999);(Motterlini et al., 2002b; Naughton et al., 2002a). The damage arises from excessive nitrosation of nucleophilic centres, thus resulting in protein modification and loss of function (Stamler and Hausladen, 1998). NO could serve as an intracellular signal to modulate the tissue stress response and the haem oxygenase pathway may interact with NO to control cellular homeostasis. It has been suggested that HO-1 could act as a feedback inhibitor of NO when the concentrations of the gaseous molecule exceed a critical threshold and the products of haem degradation might play a role in this process (Foresti and Motterlini, 1999). Indeed, exposure of various cell types including endothelial, smooth muscle, cardiac and renal cells to NO or NO species results in induction of HO-1 (Datta and Lianos, 1999; Motterlini et al., 1996a; Naughton et al., 2002b);Naughton et al., 2002b). This suggests that HO-1 induction by NO is a generalized cellular response to excessive NO production and HO-1 may be one of the cells defense mechanisms against the cytotoxic actions of high NO levels (Motterlini et al., 2002b). In addition, NO could contribute to the induction of HO-1 under reduced oxygen levels (Motterlini et al., 2000). Intriguingly, increases in iNOS activity were observed in hypoxic endothelial cells preceding HO-1 induction, suggesting endothelial HO-1 could be regulated by NO under hypoxic conditions.

NO can up-regulate HO-1 expression and haem oxygenase activity through an increase in exogenous or endogenous NO concentrations (Foresti et

al., 1997; Motterlini et al., 1996a). Therefore, NO can exert its effects not only through its short term pharmacological action but also by affecting the expression of stress responsive pathways such as HO-1, resulting in long-lasting physiological effects. NO may affect HO-1 at different levels, from mRNA to protein expression and enzymatic function. In addition, the products of haem oxygenase activity may exert feedback mechanisms on NO and NO synthase.

The HO-1 gene promoter region has a large number of diverse target DNA sequences that can bind various transcription factors. Therefore, it could be postulated that the wide variety of NO releasing agents with different chemical characteristics that can induce HO-1 may bind to their own specific areas within the HO-1 promoter region. However, an alternative mechanism for the induction of HO-1 by various NO species is that they may all inflict a single type of "nitrosative" stress which may act on a specific single transcription factor to stimulate HO-1 (Alam and Cook, 2003). For example, cardiomyocytes exposed to nitroxyl anion have shown elevated levels of the transcription factor Nrf2, which has also been found to be elevated in vascular endothelial cells exposed to NO (Naughton et al., 2002b). The transcription factor Nrf2 is an important regulator of the cellular adaptive response to oxidative stress and has a vital role in the activation of the HO-1 gene (Alam and Cook, 2003). HO-1 mRNA levels can be increased by the action of NO. Furthermore, NO can stabilize mRNA by reducing rate of mRNA turnover, therefore, NO not only enhances HO-1 gene expression but also prolongs the half-life of its mRNA (Bouton and Demple, 2000; Hartsfield et al., 1997).

1.6.10 Modulation of haem oxygenase activity by NO

NO has been demonstrated to inhibit the degradation of haem into CO, BV/BR and iron of both HO-1 and HO-2 proteins (Ding et al., 1999; Wang et al., 2003), in other words, NO is able to attenuate the activity of the enzyme. This inhibitory property of NO may form part of a feedback mechanism for haem oxygenase induction. The mechanism of inhibition differs between the two isoforms of haem oxygenase. In the HO-2 isoform, NO will directly interact with a cysteine present in the haem-binding motif of the haem oxygenase protein, with a subsequent loss of HO-2 catalytic activity (Ding et al., 1999). Conversely, NO binding to the catalytic iron atom in the HO-1 isoform has been reported to be the mechanism for inhibition of enzymic activity (Wang et al., 2003). However, the inhibition of HO-1 activity by NO is reversible, and activity is restored after removal of NO. Another possible mechanism that has been put forward for the inhibition of haem degradation is the formation of a haem-nitrosyl complex (Juckett et al., 1998). This has been supported by reports that incubation of NO with HO-1 rich microsomes resulted in a decrease in haem oxygenase activity. Vascular effects of haem oxygenase inhibitors are elevated in the absence of NO and renal CO generation is enhanced in the presence of NOS inhibitors (Rodriguez et al., 2004). However, there is vast body of contrasting reports which show that NO greatly enhances haem oxygenase activity (Foresti et al., 2003; Sammut et al., 1998), suggesting that in the long-term NO results in the enhancement of HO-1 stimulation with subsequent increase in haem metabolites. NO may exert transient inhibitory actions on haem oxygenase, but they may be tissue or organ specific or may depend on the presence of certain pathological states.

1.6.11 Interaction of NO with haem metabolites

The breakdown products of haem by HO-1 have been demonstrated to interact with NO. For example, exposure to CO gas powerfully inhibited the NOS activity, and thereby NO production, from macrophages and rat cerebellum (White and Marletta, 1992), which the authors suggest is a consequence of CO binding to the haem-containing NOS. Conversely, in a model of liver injury, inhalation of CO gas has been shown to up-regulate iNOS which in turn induced HO-1, whereby the beneficial effects of CO seemed to be mediated through NO stimulation of HO-1 (Zuckerbraun et al., 2003). Therefore, a complex interaction might exist between haemoproteins such as NOS and the haem-binding molecules CO and NO which results in the modulation of haemoprotein function.

BR and BV have been shown to interact efficiently with NO and NO related species, implicating novel 'anti-nitrosative' features for the bile pigments (Kaur et al., 2003). It has been suggested that BR and BV act as scavengers of NO, and reports have shown that interaction of bile pigments with NO species result in their degradation. However, the presence of thiols significantly prevents the loss of BR by nitroxyl ion, suggesting that the bile pigments may compete with sulphydryl groups to scavenge NO and protect cells from damage due to excessive NO (Kaur et al., 2003). Furthermore, scavenging of reactive nitrogen species have also reported to provide protection against tissue damage in an arthritic rodent model (Bezerra et al., 2004), adding support to the idea that removal of excess NO is advantageous in protecting against cellular injury.

1.6.12 Potential effect of haem and NO interaction on HO-1

A potential co-operation between NO and HO-1 has been proposed, which would result in the increased production of BV/BR (anti-oxidant), CO (anti-inflammatory and signalling) and iron (signalling), occurring in pathophysiological situations affecting vascular functions. Stamler (Stamler, 2003) and Gladwin's (Gladwin et al., 2004) work deal with NO and haemoglobin interaction at a physiological level, but there are conditions in which free haemoglobin is released into the blood to interact with NO. SCD is an example of a condition which is characterized by high concentrations of free haemoglobin, secondary to acute and chronic haemolysis, as well as conditions of hypoxia. In fact, it has been proposed that it is the scavenging of NO by free haemoglobin in SCD, thereby limiting its bioavailability, that causes the vascular complications that are SCD's pathological hallmarks (Reiter et al., 2002). Administration of NO as therapy to patients with sickle crises has been shown to ameliorate symptoms. The inherent hypoxia in SCD could also stimulate NOS and HO-1 induction (Motterlini et al., 2000), the latter occurring possibly through an enhanced interaction between NO and haemolysis-mediated free haemoglobin. These findings raise the fundamental question of the biological significance of NO and haemoglobin interaction in pathophysiological states such as SCD. If NO acts co-operatively in the presence of haemoglobin and/or haem to induce HO-1 in endothelial cells, then the HO-1 pathway could serve as an effective defensive system to the vasculature in haemolytic states. In the present study, we examined the effects of the interaction of haemoglobin and NO on endothelial haem oxygenase and investigate a possible role for this interaction in SCD.

1.7 Hypothesis and objectives

The interaction of haem and NO has previously been shown to enhance endothelial haem oxygenase induction and endothelial haem uptake (Foresti et al., 2003). This is an interesting phenomenon, as both these potentially toxic compounds can individually trigger the protective haem oxygenase pathway in endothelial cells, and their co-incubation amplifies the observed effect. This could possibly be an adaptive mechanism for cells exposed to the dual insult of excess haem and excess NO that may be present in certain haemolytic conditions such as SCD. Therefore, the general hypothesis for this thesis is:

Increased haem oxygenase activity and induction of HO-1 protein expression by interaction of haemoglobin and NO is an adaptive response of the endothelium and may have a physiological role in conditions such as sickle cell disease.

To verify this theory a number of objectives were set:

- to investigate the effect of NO and haemoglobin interaction on endothelial haem oxygenase induction
- to examine the effect of NO and haemoglobin interaction on endothelial haem uptake
- to explore the effect of sickle blood on endothelial haem oxygenase induction
- to examine the effect of haem oxygenase and its breakdown products on sickle blood interaction with vascular endothelium
- to study the effect of HO-1 inducers on erythroid progenitor cells (K562)

Chapter 2. Materials and Methods

2.1 Reagents

Human haemoglobin-A_0 ferrous stabilized (HbA_0), human sickle cell haemoglobin (HbS), human methaemoglobin (MetHb), hydroxyurea (HU), curcumin, thymidine and media and cell culture media supplements (L-glutamine, foetal bovine serum, and bovine serum albumin) were obtained from Sigma. Foetal haemoglobin (HbF) was purchased from Bethyl laboratories. Streptomycin, penicillin and Dulbecco's phosphate buffered saline were purchased from Invitrogen. Haemin, biliverdin (BV) and tin protoporphyrin IX (SnPPIX) were purchased from Porphyrin Products (Logan, UT, U.S.A.). S-nitrosoglutathione (GSNO), Angeli's Salt (AS), (Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO), 2-(N,N-Diethylamino)diazenolate-2-oxide sodium salt (DEA-NO) and 1-propanamine,3-(2-hydroxy-2-nitroso-1-propylhydrazino) (PAPA-NO) were obtained from Alexis Corporation (Bingham, UK). The nitrosyl adduct of haemin (haem-nitrosyl or NO-Fe^{II}-protoporphyrin IX) and CO-RMs (CORM-3 and CORM-A1) were prepared as described below. All other reagents were purchased from Sigma unless otherwise stated.

2.2 Synthesis of ferrous haem-nitrosyl (H-NO)

Ferrous haem-nitrosyl was prepared by our collaborators Dr. Patrick J. Farmer and Filip Sulc (University of California, Irvine, California). All experiments were conducted under strict anaerobic conditions by using standard Schlenk techniques. The nitrosyl adduct of haemin (NO-Fe^{II}-protoporphyrin IX) was generated by dissolving haemin (Aldrich) in carbonate

buffer (pH=10), addition of 40-fold excess of sodium dithionite (Fisher) followed by addition of 20-fold excess of sodium nitrite (Sigma) under anaerobic conditions (Figure 2-1). During the reaction the solution turned from brown to deep red, indicating the formation of the ferrous nitrosyl adduct. The pH was adjusted to 7 by addition of excess buffer, and the nitrosyl haemin was extracted into methyl ethyl ketone. The extract was washed anaerobically with buffer (pH=7) three times, and the solution dried to an air stable solid. Re-dissolution of the solid in buffer (pH=10) allowed spectral characterization of an intense Soret maxima at 420 nm, confirming the formation of the ferrous nitrosyl adduct. The electronic spectra were recorded on a Hewlett-Packard 8453 spectrophotometer. Since the haem-nitrosyl adduct has limited stability in aerobic solutions, ultimately forming nitrate and ferric haemin, great care was taken to reduce the exposure of the complex to air before the experiments

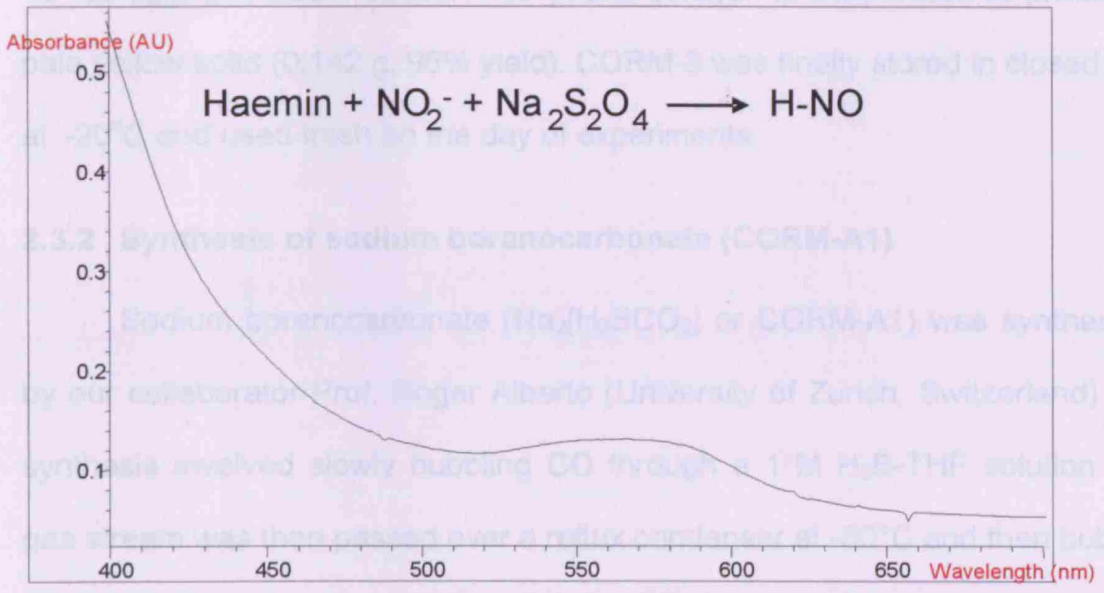


Figure 2-1. Spectrum of H-NO generation

Independent generation of H-NO by standard method: excess sodium nitrite and disodium dithionite were added to the haemin solution in pH 7.4 phosphate buffer, as described. The peak of absorbance of H-NO is 420 nm.

2.3 Synthesis of CO-releasing molecules (CO-RMs)

2.3.1 Synthesis of tricarbonylchloro(glycinato)ruthenium (II) (CORM-3)

Tricarbonylchloro(glycinato)ruthenium(II) ($[Ru(CO)_3Cl(glycinate)]$ or CORM-3) was synthesised by our collaborator Prof. Brian. E. Mann (University of Sheffield, Sheffield, UK). The synthesis of CORM-3 starts with the use of a commercially available compound, tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$) (Sigma, Aldrich). Briefly, ($[Ru(CO)_3Cl_2]_2$) (0.129 g) and glycine (0.039 g) were placed under nitrogen in a round-bottomed flask. Methanol (75 ml) and sodium ethoxide (0.034 g) were added and the reaction allowed to continue under stirring for 18 h at room temperature. This was followed by removal of the solvent under pressure and the remaining yellow residue redissolved in tetrahydrofuran (THF), which was then filtered and an excess of 40- 60 light petroleum added. This yellow solution is evaporated to produce a pale yellow solid (0.142 g, 96% yield). CORM-3 was finally stored in closed vials at -20°C and used fresh on the day of experiments.

2.3.2 Synthesis of sodium boranocarbonate (CORM-A1)

Sodium boranocarbonate ($Na_2[H_3BCO_2]$ or CORM-A1) was synthesised by our collaborator Prof. Roger Alberto (University of Zurich, Switzerland). The synthesis involved slowly bubbling CO through a 1 M H_3B-THF solution. The gas stream was then passed over a reflux condenser at -50°C and then bubbled through a solution of NaOH in ethanol in a Schlenk tube at -78°C. After 2 h the Schlenk tube was disconnected and heated to reflux for 1 h. CORM-A1 precipitated as a white solid and was filtered and washed with cold ethanol and

diethyl ether to yield the pure product. CORM-A1 was finally stored in closed vials at -20°C and used fresh on the day of experiments.

2.3.3 Release of CO from CORM-3 and CORM-A1

Detection of CO release from CORM-3 and CORM-A1 was confirmed in the laboratory using a myoglobin assay (Figure 2-2).

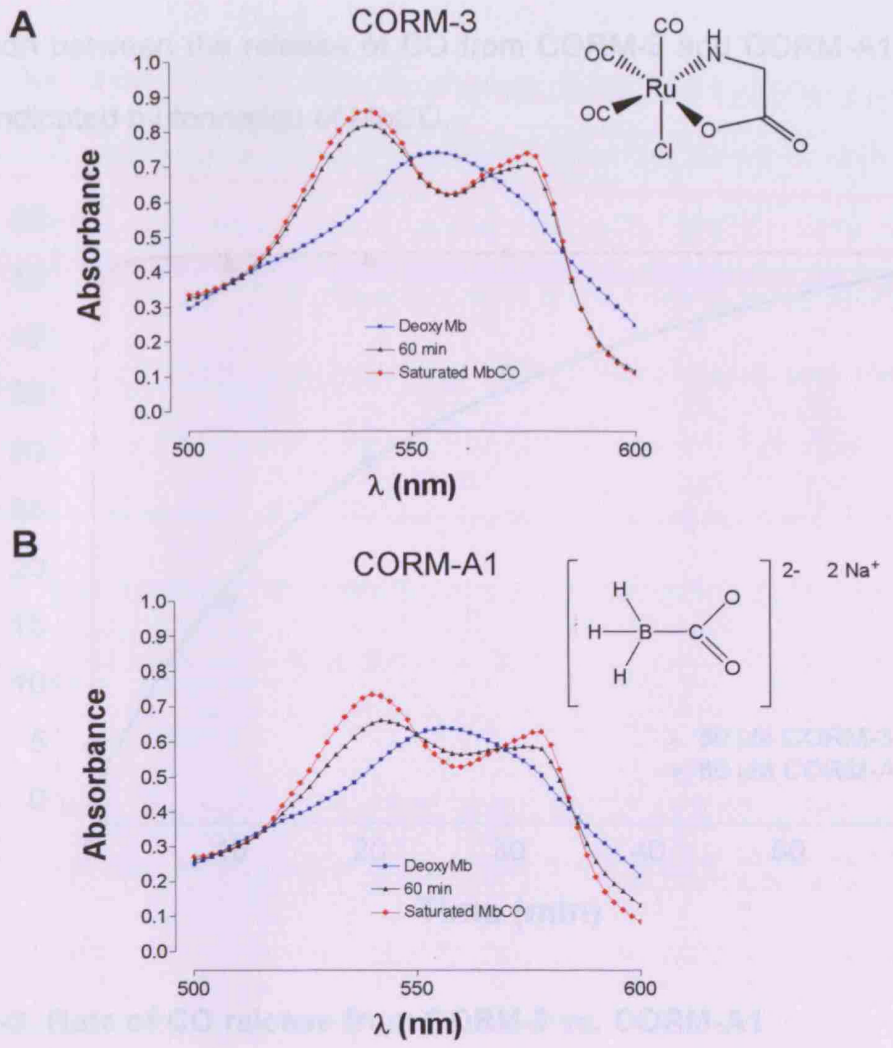


Figure 2-2. Release of CO from CORM-3 and CORM-A1 in aqueous solutions

(A) Chemical structure of $[Ru(CO)_3Cl(glycinate)]$ (CORM-3) and detection of carbonmonoxy myoglobin (MbCO) formation after addition of 40 µmol/l CORM-3 to a deoxymyoglobin (deoxyMb) solution. (B) Chemical structure of $Na_2[H_3BCO_2]$ (CORM-A1) and detection of MbCO as for CORM-3 after addition of 40 µmol/l CORM-A1.

This assay consists of measuring spectrophotometrically by measuring the conversion of deoxymyoglobin (deoxy-Mb) to carbonmonoxy myoglobin (MbCO) after addition of our test molecules. CORM-3 and CORM-A1 are both water soluble compounds, but possess different chemical characteristics that affect their rate of CO release (CORM-3 has a half-life of 3.6 min, while CORM-A1 has a half-life of 21 min in physiological buffers). Figure 2-3 shows a comparison between the release of CO from CORM-3 and CORM-A1 over an hour as indicated by formation of MbCO.

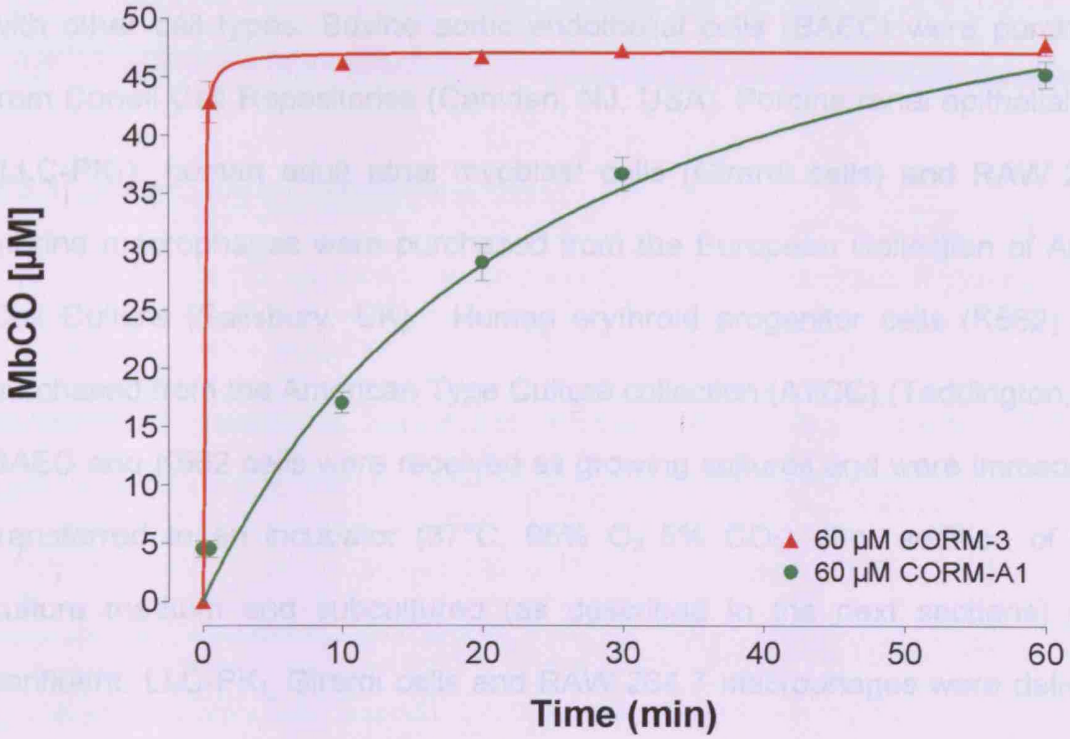


Figure 2-3. Rate of CO release from CORM-3 vs. CORM-A1

Detection of carbonmonoxy myoglobin (MbCO) after addition of 60 µmol/l CORM-3 or CORM-A1 to a deoxymyoglobin (deoxy-Mb) solution. CORM-A1 slowly releases CO, reaching MbCO saturation after 1 h, whereas CORM-3 has reached saturation after 1 min.

Inactive CO-RMs or iCORMS which were stimulated to release all their CO were used as negative controls. iCORM-3 was generated by dissolving CORM-

3 in Krebs buffer (pH 7.4) and left overnight at room temperature to become inactive. iCORM-A1 was generated by initially dissolving CORM-A1 in 0.1 M HCl and bubbling pure N_2 through the solution for 10 min to remove any residual CO gas.

2.4 Cell culture

Commercially available cell lines were used for the work reported in this thesis rather than primary cultures as they were more stable, less labour intensive, had longer life-spans and did not have problems of contamination with other cell types. Bovine aortic endothelial cells (BAEC) were purchased from Coriell Cell Repositories (Camden, NJ, USA). Porcine renal epithelial cells (LLC-PK_1), human adult atrial myoblast cells (Girardi cells) and RAW 264.7 murine macrophages were purchased from the European Collection of Animal Cell Culture (Salisbury, UK). Human erythroid progenitor cells (K562) were purchased from the American Type Culture collection (ATCC) (Teddington, UK). BAEC and K562 cells were received as growing cultures and were immediately transferred to an incubator (37°C, 95% O_2 5% CO_2) after addition of fresh culture medium and subcultured (as described in the next sections) when confluent. LLC-PK_1, Girardi cells and RAW 264.7 macrophages were delivered frozen in cryoprotectant (DMSO). Upon receipt, cells were immediately thawed in a 37°C water bath, the cryoprotecant containing medium discarded and fresh medium added (Table 2-1 summarises the composition of culture medium for specific cell types). Cells were transferred to a 25 cm^2 flask (Sarstedt Ltd., Leicester, UK) and incubated at 37°C in a 95% O_2, 5% CO_2 humidified atmosphere. Fresh medium was added every two days to the flask, and cells

were subcultured once confluent. The following sections describe the specific subculture of each cell type in more detail.

Table 2-1. Culture medium of different cell types

Cell type	Media	FBS (%)	L-glutamine (mM)	Penicillin-Streptomycin(U/ml)	Additives
BAEC	Iscove's	10	2 mM	100	-
LLC-PK_1	DMEM	10	2 mM	100	-
Girardi	DMEM	10	3.5 mM	100	NEAA*
Raw267.4	DMEM	10	2 mM	100	-
K562	Iscove's	10	3.5 mM	100	-

* NEAA=non-essential amino acids (Sigma,UK)

2.4.1 Bovine aortic endothelial cells

BAEC were cultured in Iscove's modified Dulbecco's medium with supplements as described in Table 2-1. To maintain the cell line for experiments, cells were subcultured approximately every 3 days. Once the cells reached confluence (>80% coverage of 75 cm^2 flask (Sarstedt Ltd., Leicester, UK), they were washed with 5 ml of warm (37°C) PBS (Gibco, Paisley, UK) to remove any residual media before addition of trypsin (0.25% trypsin-EDTA solution, Sigma) for approximately 3 min at 37°C. Trypsin and cells resuspended in the solution were then removed and pipetted into 8 ml of fresh medium to neutralize its action. Cells were centrifuged (1500 x *g*) for 5 min in bench top centrifuge (SANYO, Harrier 15/80). The cell pellet was then resuspended in fresh medium and split (1:3) into preprepared 75 cm^2 flasks containing fresh medium (8 ml). Cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere and when the cells reached 80-90% confluence they

underwent experimental protocol. All cell culture work was carried out using an ESB JBIO CL II category II hood.

2.4.2 Porcine renal epithelial cells (LLC-PK_1)

Porcine renal epithelial cells were grown in Dulbecco's modified Eagle's medium (DMEM) with supplements as described in Table 2-1. To maintain the cell line for experiments, cells were subcultured approximately every 3 days as described above.

2.4.3 Human adult atrial myoblast cells (Girardi)

Girardi cells were cultured in DMEM with supplements as described in Table 2-1. To maintain the cell line for experiments, cells were subcultured as previously described.

2.4.4 RAW 264.7 murine macrophages

RAW 264.7 macrophages were grown in DMEM with supplements as described in Table 2-1. These cells were subcultured by removing the old medium and washing cells with 5 ml warm (37°C) PBS, which was discarded and replaced with 8 ml warm (37°C) DMEM. Cells were then scraped from the surface of the flask with a rubber plastic Thomas policeman, pipetted into prepared 75 cm^2 flasks (each containing 8 ml DMEM) and returned to the incubator.

2.4.5 Human erythroid progenitor cells (K562)

K562 cells were cultured in Iscove's modified Dulbecco's medium with supplements as described in Table 2-1. To maintain the cell line for experiments, cells were subcultured approximately every 3 days. Subculturing

of these non-adherent cells involved centrifugation of cells in old medium (100 x *g*) for 5 min followed by resuspension of the cell pellet in 8 ml fresh medium to split cells 1:4. 75 cm^2 flasks were pre-prepared with 8 ml fresh media. Cells were treated when cultures had reached 1 x 10^5 viable cells/ml as determined using a haemocytometer.

2.4.6 Subculture of cells for incubation

Cells were cultured in 75-cm^2 flasks for haem oxygenase activity and Western blot analysis. For determination of haem content, cells were cultured in 24-well dishes (Fisher, UK), for the BR assay cells were cultured in 35 mm diameter Petri dishes (Fisher, UK) and for the cell adhesion assay cells were cultured in 6-well plates (Fisher, UK).

2.5 Collection of samples

Following each experimental treatment, cells were washed once with 5 ml of cold (4°C) PBS followed by addition of 5 ml of cold (4°C) PBS to the flasks. Cells were gently scraped with a rubber Thomas policeman and the cell suspension was then transferred to a 15 ml tube (Sarstedt, Leicester, UK) and a further 4 ml PBS was added to the flask to collect any remaining cells. Samples were centrifuged for 10 min at 1800 g at 4°C, the supernatant was discarded and the pellet was resuspended in either: 1) 100 mM PBS – 2 mM $MgCl_2$ buffer (550 µl) for the haem oxygenase activity assay or, 2) 1% Triton PBS (250 µl) for Western blot Analysis. All samples were stored at -80°C until assays were performed.

2.6 Preparation of liver cytosol and liver microsomes

Liver cytosol was prepared from male Sprague Dawley rats (250-300 g). After sacrifice by anaesthetization (pentobarbitone Lethobarb sodium BP, 500 µl, i.p.) and cervical dislocation, a 50 ml syringe filled with cold (4°C) buffer (1.15% (w/v) KCl) was used to perfuse through the liver lobes and remove any residual blood, while clotted tissue was discarded. The organ was weighed, finely chopped with a scissors and homogenised in 2-3 volumes of homogenising buffer (20 mM Tris-HCl, pH 7.4, containing 1.15% (w/v) KCl), followed by partition among a set of polyallomer centrifuge tubes (Beckman Coulter Ltd., High Wycombe, UK). Following centrifugation (5,000 x *g*) for 20 min at 4°C in a refrigerated ultracentrifuge (Optima LE 80, Beckman Instruments, Palo Alto, California, USA), the supernatant was retained and centrifuged again (105,000 x *g*) for 90 min at 4°C. Following each centrifugation, the lipid layer was removed with a Pasteur pipette. The supernatant was collected, and biliverdin reductase activity (using a modified haem oxygenase assay) and protein were measured as described in Sections 2.7 and 2.8, respectively, then aliquoted and stored at -70°C.

The preparation of liver microsomes is similar to that for liver cytosol with the following variations. The rat was pre-treated with haemin (50 mg/kg, i.p.) 24 h beforehand to induce haem oxygenase activity. The lobes were perfused with cold (4°C) saline (0.9% (w/v) NaCl) and homogenised in 5 volumes of sucrose solution (0.25 M sucrose, 0.05 M Tris-HCl, pH 7.4) and then centrifuged as described above. After the last centrifugation step, the resulting microsomal pellet was resuspended gently in 1 ml of phosphate buffer (pH 7.4) and haem

oxygenase activity and protein were determined according to Sections 2.7 and 2.8, respectively, followed by storage at -70°C.

2.7 Assay for endothelial haem oxygenase activity

Haem oxygenase activity was determined in endothelial cells at different times after treatment. The stock solutions of the assay reagents (i.e. glucose-6-phoshate (G6P) (20 mM), glucose-6-phoshate dehydrogenase (G6PDH) (50 U/ml) and NADPH (40 mM)) were prepared with phosphate buffer (pH 7.4) and stored at -80°C until required. The NADPH stock solution was kept wrapped in foil as it is light sensitive. Haemin stock (2 mM) was prepared fresh for each assay by first solubilising the haemin in a small amount of 0.5% (v/v) NaOH (2 M) and then making up the stock in phosphate buffer (PBS-$MgCl_2$, pH 7.4). Rat liver cytosol and liver microsomes were prepared according to Section 2.6. Collection of samples was as described in section 2.5. Cells were lysed by three rounds of freeze-thawing (-80° to 37°C), then placed on ice. Four hundred µl of the resulting cell suspension was added to a set of glass tubes, which were maintained on ice (a 100 µl aliquot of the remaining cell suspension was retained for protein determination according to Section 2.8). A reaction mixture was added to the cell suspension (final volume, 900 µl) in the following order: PBS-$MgCl_2$ (pH 7.4), 20 µM haemin, 2 mM G6P, 0.5 U/ml G6PDH, 3 mg of rat liver cytosol as a source of biliverdin reductase, and 0.8 mM NADPH. The negative (PBS-$MgCl_2$, pH 7.4) and positive (rat liver microsomes) controls were also prepared, containing proportionate volumes of phosphate buffer (pH 7.4) instead of the cell suspension. The tubes containing the cell suspension/reaction mixtures were vortexed well and incubated in the dark for 1 h at 37°C. Activity of the cytosolic and microsomal fractions was verified by

replacing haemin with BV (1 mM), while the samples were incubated in the dark for 30 min at 37°C. After 1 h incubation, the reaction was terminated by addition of chloroform (1 ml), followed by thorough vortexing and centrifuging (1800 x *g*) 2 times for 5 min at room temperature until three distinct layers were formed. Using a quartz cuvette and the UVikon 810P spectrophotometer the absorbance (at 464 nm and 530 nm) of the lower organic layer, which contains BR (extinction coefficient (ε) for BR in chloroform, 40 $mM^{-1}cm^{-1}$), was read against a blank of chloroform. Haem oxygenase activity was expressed as picomoles of BR formed/mg protein/h using the formula:

$$\frac{\text{pmoles bilirubin}}{\text{mg protein in 60 min}} = \left(\frac{\Delta OD\ (OD_{464} - OD_{530})}{40} \Big/ \text{mg protein} \right) \times 10^6$$

Equation 1. Calculation of haem oxygenase activity

2.8 Protein determination

Protein concentration in samples was determined using the DC Protein Assay kit (Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK). The assay is similar to the Lowry assay and is based on protein reacting with an alkaline copper tartrate solution and Folin reagent. The colorimetric assay depends on the colour development after reaction between protein and copper in an alkaline medium, and a subsequent reduction of Folin reagent by the copper-treated protein. The presence of protein results in the production of a blue colour, the more intense the blue the higher the level of protein present. A standard curve was prepared using serial dilutions (0 to 2.8 mg/ml) of a bovine serum albumin (BSA) standard (Bio-Rad Laboratories Ltd.), prepared in triplicate, in phosphate buffer (pH 7.4). The absorbance readings at an optical density of 750 nm (OD_{750}) were plotted against their respective protein standard

concentrations. The procedure for protein determination in cell and tissue samples was performed as follows. A 100 µl aliquot of the cell suspension (sample preparation as described in 2.4) and a blank of phosphate buffer or PBS Triton X-100 were added to 10 ml polystyrene tubes (Sarstedt Ltd.). To each tube, 500 µl of A' (20 µl of Reagent S per ml of Reagent A) was added, then vortexed briefly. The next step was the addition of 4 ml of Reagent B to each tube, which was mixed well and then left at room temperature for 20 min before reading the OD_{750} against the blank using an UVikon 810P spectrophotometer (Tegimenta AG, Switzerland). A numerical value (mg/ml) for the protein concentration in the unknown samples was derived by multiplying the respective OD_{750} by the slope of the standard curve (Figure 2-4).

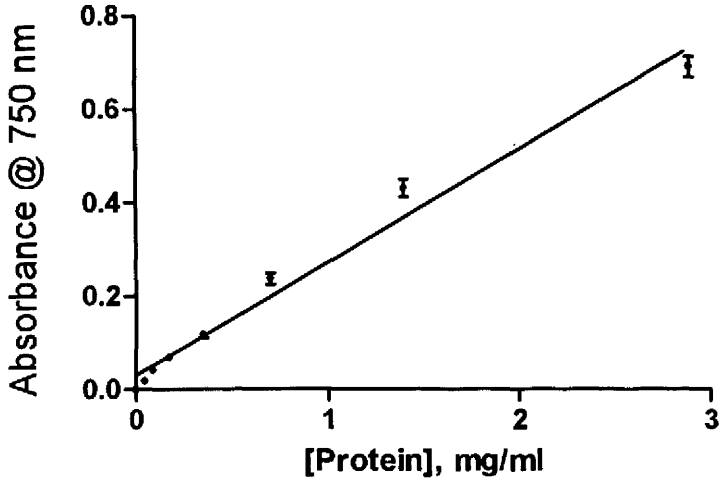


Figure 2-4. Representative standard curve for protein determination

2.9 Western-blot technique for detection of HO-1 protein expression

Samples of endothelial cells also underwent Western immunoblot analysis. Cell samples were prepared as described in section 2.5, and protein levels of each sample were calculated as mentioned in 2.8. Appropriate volumes of each

sample were combined with loading buffer (Laemmli buffer, Bio-Rad Laboratories Ltd.) containing 5% (v/v) β-mercaptoethanol) to a give a total volume of 30 µl and a final protein concentration loaded onto each lane equal to 30-50 µg. The protein levels of β-actin, a housekeeping gene, were also measured by Western immunoblot analysis to ensure equal loading of cell samples. Therefore the densitometric analyses of the HO-1 protein bands are expressed as HO-1/β-actin ratio to normalize HO-1 protein levels.

The HO-1 positive control (Bioquote Ltd., York, UK) was diluted to 1 µg/ml in loading buffer. Samples were pulsed for 20-30 sec on a bench-top microcentrifuge (MSE Microcentaur, Sanyo Gallenkamp Plc., Leicestershire, UK) followed by denaturing of the proteins for 10 min at 100°C on a heating block (Techne DB2A, Techne GmBH, Germany). The molecular weight marker (MWM, Invitrogen LifeTechnologies Ltd.) was not heated but placed on ice until it was required. After heating the samples were re-pulsed and 30 µl of samples, 10 µl MWM and positive control were loaded carefully into the wells of 12% tris-glycine Ready gel (Bio-Rad Laboratories Ltd.) using gel loading tips (Fisher, UK). Electrophoresis was carried out at room temperature in a tank containing running buffer (0.025 M Tris, 0.192 M glycine, 0.1% (v/v) SDS) (Fischer, UK) using the Mini-PROTEAN® II system and Power-Pac 300 power supply (Bio-Rad Laboratories Ltd.). A constant voltage of 125 V was applied until the loading buffer had migrated to, but not beyond, the base of the gel.

The subsequent procedures were carried out using the Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad Laboratories Ltd.). While the gel was running, sheets of nitrocellulose membrane 45 µm pore size (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) and 3MM Whatman

blotting paper were cut and pre-soaked in distilled H_2O and cold (4°C) transfer buffer (0.025 M Tris, 0.192 M glycine, 0.1% (v/v) SDS, 20% (v/v) methanol) for 10 and 5 min, respectively. After electrophoresis was completed a 'gel sandwich' (Figure 2-5) was prepared upon the clear (positive electrode) side of a gel cassette. After removing any air bubbles the cassette was closed securely via the latch and placed into the electrode module with the black (negative electrode) side facing the black panel of the module. This orientation ensures the proteins migrate from the gel onto the nitrocellulose membrane and not into the transfer buffer while transferring. Overnight transfer was carried out at 4°C with a constant voltage of 30 V using a 1000/500 transfer unit from Bio-Rad Laboratories Ltd.

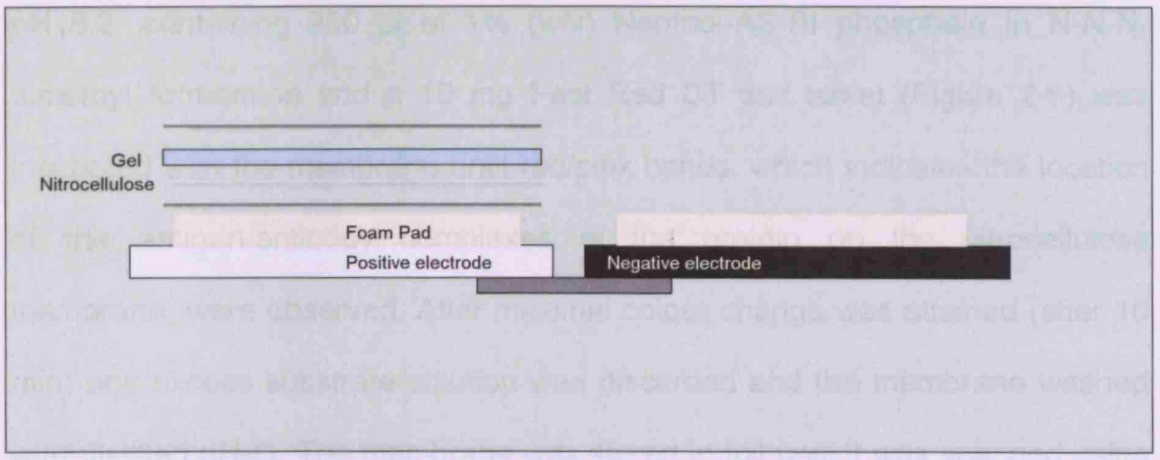


Figure 2-5. Arrangement of gel sandwich assembly

All subsequent procedures were performed at room temperature on an orbital shaker (Denley Instruments Ltd., Sussex, UK) at low speed. Non-specific binding of the antibodies was prevented by incubating the membrane in blocking solution containing non-fat dried milk (5% (w/v)) in PBS (0.01 M phosphate buffer, pH 7.4, containing 0.2% Tween-20) for 2 h. This was followed by a single 5 min wash with 10 ml PBS (pH 7.4). The membrane was incubated

for 2 h with anti-HO-1 (Bioquote Ltd., York, UK) diluted 1:1000 in Tris buffered saline (TBS) (0.05 M Tris-HCl, 0.0037 M KCl, 0.7137 M NaOH, pH 7.4). Next, the membrane was washed three times, once with PBS-T (PBS 0.01 M, pH 7.4, containing 0.05% (v/v) Tween 20), then twice with TBS (pH 7.4).

Proteins were visualised using an ExtrAvidin® alkaline phosphatase staining kit (Sigma), according to the following procedure. The biotinylated anti-rabbit IgG antibody, diluted 1:1000 in TBS (pH 7.4), was incubated with the membrane for 1 h. The membrane was washed three times with TBS (pH 7.4) and the ExtrAvidin® alkaline phosphatase conjugate, diluted 1:1000 in TBS (pH 7.4), applied for 1 h. The three washes with TBS (pH 7.4) were repeated. After the last wash a freshly prepared substrate solution (9.8 ml of 0.1 M Tris buffer, pH 8.2, containing 200 µl of 1% (w/v) Napthol-AS-BI phosphate in N-N-N-dimethyl formamide and a 10 mg Fast Red DT salt tablet (Figure 2-6) was incubated with the membrane until red/pink bands, which indicates the location of the antigen-antibody complexes of the protein on the nitrocellulose membrane, were observed. After maximal colour change was attained (after 10 min) any excess substrate solution was discarded and the membrane washed with distilled dH_2O. The membrane was stored in foil until it was scanned using the Photoshop software.

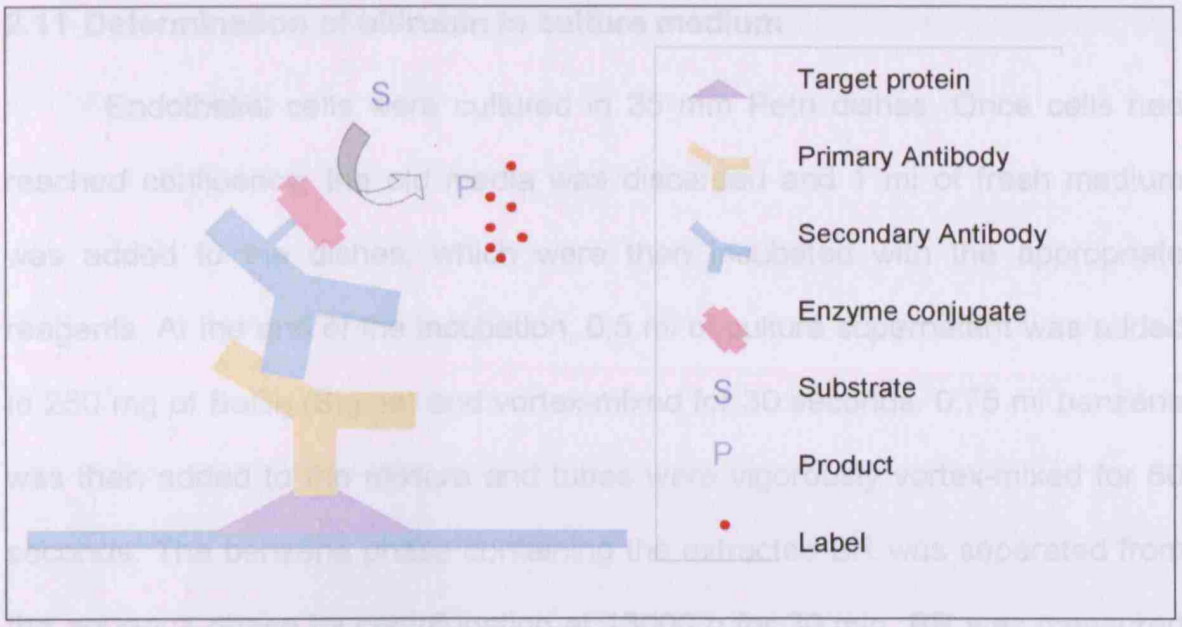


Figure 2-6. Binding cascade for development of bands for HO-1 Western blot

2.10 Assay for determination of cellular haem content

Endothelial cells were grown in 24 well dishes until they reached confluency. The medium in each well was replaced with fresh medium and after the appropriate reagents were added to each well the dishes were returned to the incubator for various incubation times. At the end of the incubation the medium was removed and cells were washed twice with warm (37°C) PBS, followed by addition of 1 ml formic acid (95-97%, Sigma) to solubilise the endothelial layer. The haem concentration in the formic acid solution was measured spectrophotometrically at 398 nm ($\varepsilon = 1.56 \times 10^5\ M^{-1}cm^{-1}$) using a UVikon 810P spectrophotometer. Intracellular haem concentration was expressed as pmol/well.

2.11 Determination of bilirubin in culture medium

Endothelial cells were cultured in 35 mm Petri dishes. Once cells had reached confluency, the old media was discarded and 1 ml of fresh medium was added to the dishes, which were then incubated with the appropriate reagents. At the end of the incubation, 0.5 ml of culture supernatant was added to 250 mg of $BaCl_2$ (Sigma) and vortex-mixed for 30 seconds. 0.75 ml benzene was then added to the mixture and tubes were vigorously vortex-mixed for 60 seconds. The benzene phase containing the extracted BR was separated from the aqueous phase by centrifugation at 13000 *g* for 30 min. BR was measured spectrophotometrically as a difference in absorbance between 450 and 600 nm (ε = 27.3 $mM^{-1} \cdot cm^{-1}$) using a UVikon 810P spectrophotometer and was expressed as nM/mg protein. As benzene is a hazardous chemical, all appropriate safety measures were adhered to when performing the assay. A potential alternative to benzene is chloroform which, although possessing lower sensitivity for the measurement of BR, may be is less toxic than benzene. However, the same safety control measures undertaken when using benzene should also be applied for chloroform.

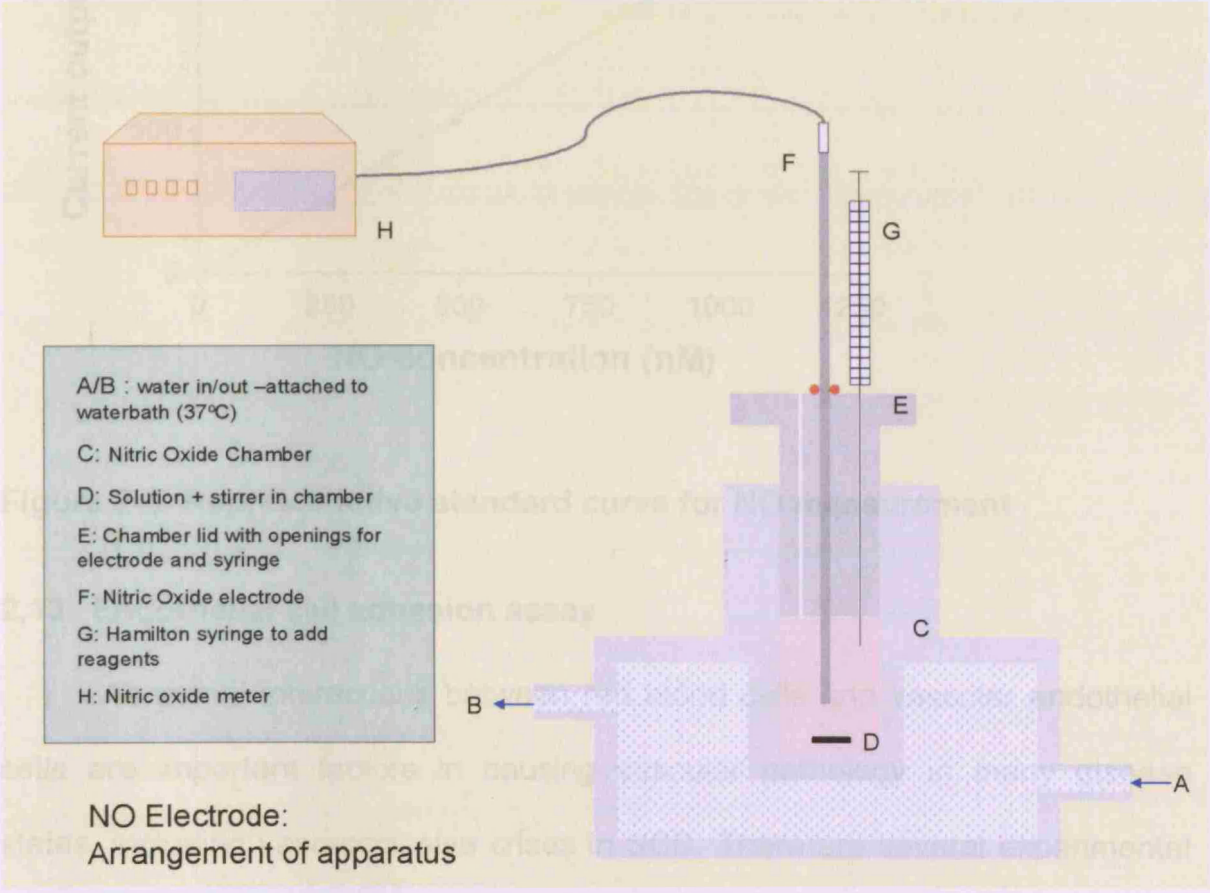
2.12 Nitric oxide electrode

The NO electrode can be used to measure the release of NO into physiological solutions. We used the NO electrode to investigate the interaction of NO donors and haemoglobins in cell culture medium and to observe the effects of haemoglobins on NO release by NO donors. The NO chamber (NOCHM, W.P.I., Sarasota USA), NO electrode (ISO-NOP, W.P.I., Sarasota USA) and NO meter (ISO-NOTM Mark II, W.P.I., Sarasota USA) were set up as shown in Figure 2-7. The NO electrode was calibrated using chemical

generation of NO by potassium nitrite. The method of calibration is based on the following reaction:

$2KNO_2 + 2KI + 2H_2SO_4 \rightarrow 2NO + I_2 + 2H_2O + 2K_2SO_4$

The following calibration solutions were prepared: Solution 1 – 0.1 M H_2SO_4 + 1.0 M KI and Solution 2 – 50 µM KNO_2. To calibrate the NO electrode a 2 ml volume of Solution 1 was placed in the NO chamber, and known concentrations of KNO_2 were added to generate a known amount of NO.



NO Electrode:
Arrangement of apparatus

Figure 2-7. NO electrode experimental set-up

The ratio between KNO_2 and NO is 1:1, therefore the amount of NO generated in the solution will be equal to the amount of KNO_2 added. Standard concentrations of NO are generated to produce a standard curve (Figure 2-8) and calibrate the NO electrode. Once the NO electrode is calibrated, 1 ml of medium (Iscoves's medium used with endothelial cells for haem oxygenase

activity and HO-1 expression experiments) is placed in the NO chamber and the NO electrode was allowed to equilibrate until the baseline reading was steady and ready for experimentation.

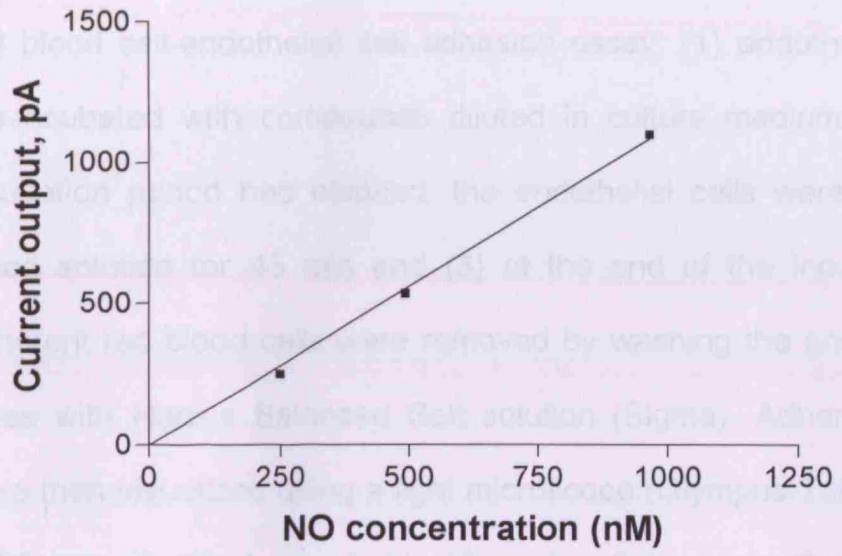


Figure 2-8. Representative standard curve for NO measurement

2.13 Endothelial cell adhesion assay

Abnormal interactions between red blood cells and vascular endothelial cells are important factors in causing vascular pathology in many disease states, including vaso-occlusive crises in SCD. There are several experimental models that are commonly used to explore red blood cell-endothelial cell interactions. We used an *in vitro* static assay (Gravity sedimentation method), as this method allowed for fine control over biochemical and biophysical variables. Firstly, endothelial cells were subcultured into 6-well dishes (endothelial cell culture protocol as described in section 2.4.1). Preliminary experiments were designed to determine the optimal percentage of blood

solution needed for incubation and the length of exposure to blood required to allow for adequate red blood cell-endothelial cell interaction (see section 6.3.1 for details). It was found that incubation of endothelial cells for 45 min with a 5% whole blood solution prepared in culture medium were the optimal conditions to obtain reproducible results. Figure 2-9 shows a summary of the red blood cell-endothelial cell adhesion assay: (1) endothelial cells were first pre-incubated with compounds diluted in culture medium; (2) after the pre-incubation period had elapsed, the endothelial cells were exposed to whole blood solution for 45 min and (3) at the end of the incubation period non-adherent red blood cells were removed by washing the endothelial layer three times with Hank's Balanced Salt solution (Sigma). Adherent red blood cells were then visualized using a light microscope (Olympus Tokyo CK microscope) (x 20 magnification), counted in 10 random fields and adhesion was expressed as the number of adherent red blood cell/mm^2.

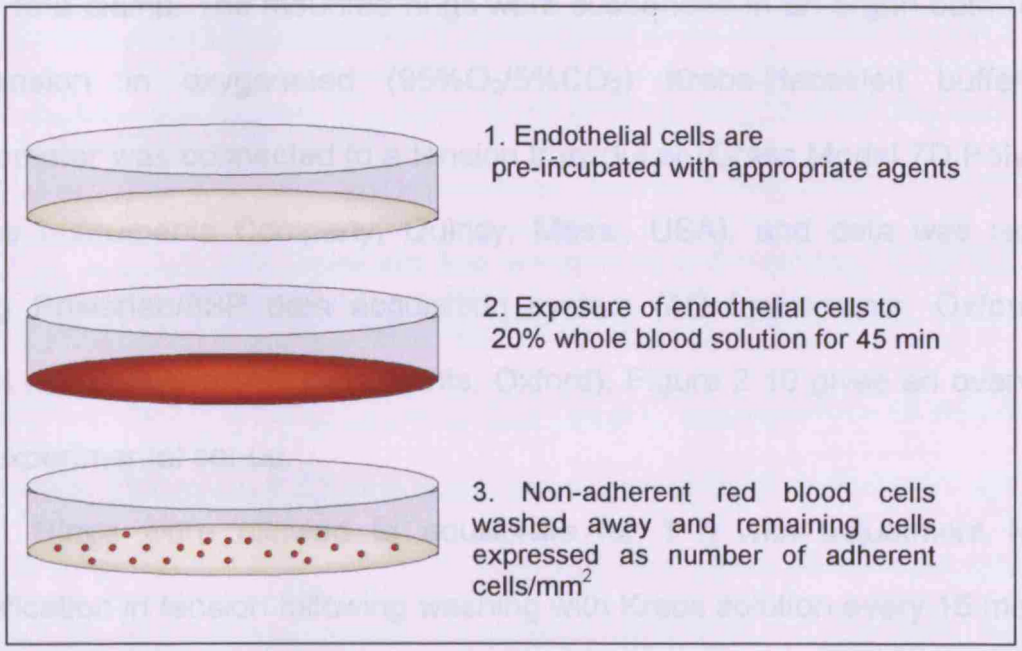


Figure 2-9. Summary of red blood cell-endothelial cell adhesion assay protocol

2.14 Isolated rat aortic ring

The isolated rat aortic ring model was used to assess the vasorelaxant effects of CO-RMs and NO donors in aortic rings exposed to sickle or normal blood. Male adult Sprague-Dawley rats were sacrificed by cervical dislocation. Animals were exsanguinated, the thorax opened and the descending aorta removed and placed into a Petri dish containing ice-cold Krebs-Henseleit buffer (mM): (NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 22, glucose 11, K^+EDTA 0.03, $CaCl_2$ 2.5). Indomethacin (10 μM) was also present in the buffer to remove the possible effect of prostaglandins on vascular tone. The lumen of the vessel was flushed gently with ice-cold buffer to remove any possible remaining blood. The aorta was carefully cleaned of fat and connective tissue and then cut into rings of 2-5 mm width. Each ring was mounted onto stainless steel hooks, with one hook being attached to a micrometer (236M series Micrometer, The L.S.Starret company, Mass., USA) and the other hook fixed to a clamp. The mounted rings were suspended in an organ bath under 2 g tension in oxygenated ($95\%O_2/5\%CO_2$) Krebs-Henseleit buffer. The micrometer was connected to a tension transducer (Grass Model 7D Polygraph, Grass Instruments Company, Quincy, Mass., USA), and data was recorded using Powerlab/8SP data acquisition system (AD Instruments, Oxford) and Chart 4.2 Software (AD instruments, Oxford). Figure 2-10 gives an overview of the experimental set-up.

Rings were allowed to equilibrate for 1 h with adjustment for any modification in tension following washing with Krebs solution every 15 min. The rings were then contracted with KCl (100 mM) and 1 μM phenylephrine before the start of any treatment.

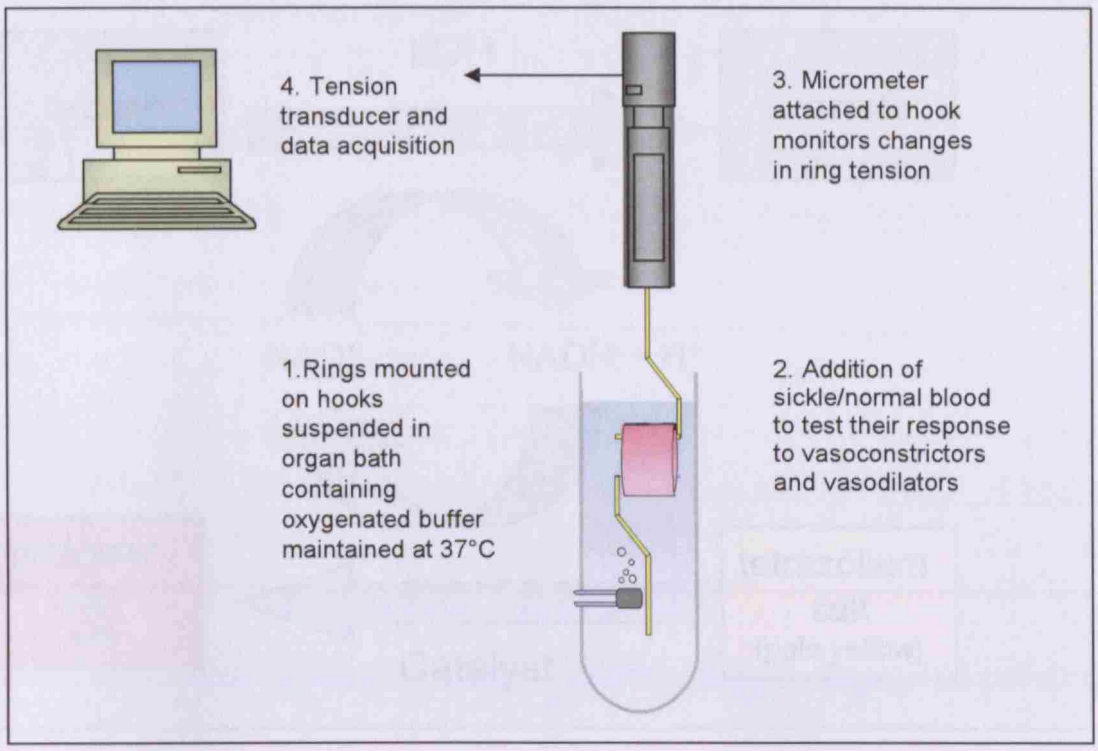


Figure 2-10. Overview of isolated aortic ring experimental set-up

2.15 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells and it is rapidly released into the cell culture supernatant upon damage of the plasma membrane. The LDH assay (Roche Diagnostics, UK) is based on the measurement of cytoplasmic enzyme activity released by damaged cells. There is a correlation between the amount of LDH activity detected in the culture supernatant and the proportion of lysed cells. For LDH detection, 100 µl of culture supernatant was collected cell-free and incubated for 15 min with 100 µl reaction mixture in a 96 well plate, according to manufacturer's instructions. LDH activity was determined in an enzymatic test: firstly, NAD^+ was reduced to NAD/H^+ by the LDH-catalysed conversion of lactate to pyruvate. This was followed by the catalyst (diaphorase) transferring H/H^+ to the tetrazolium salt INT which is reduced to formazan (Figure 2-11).

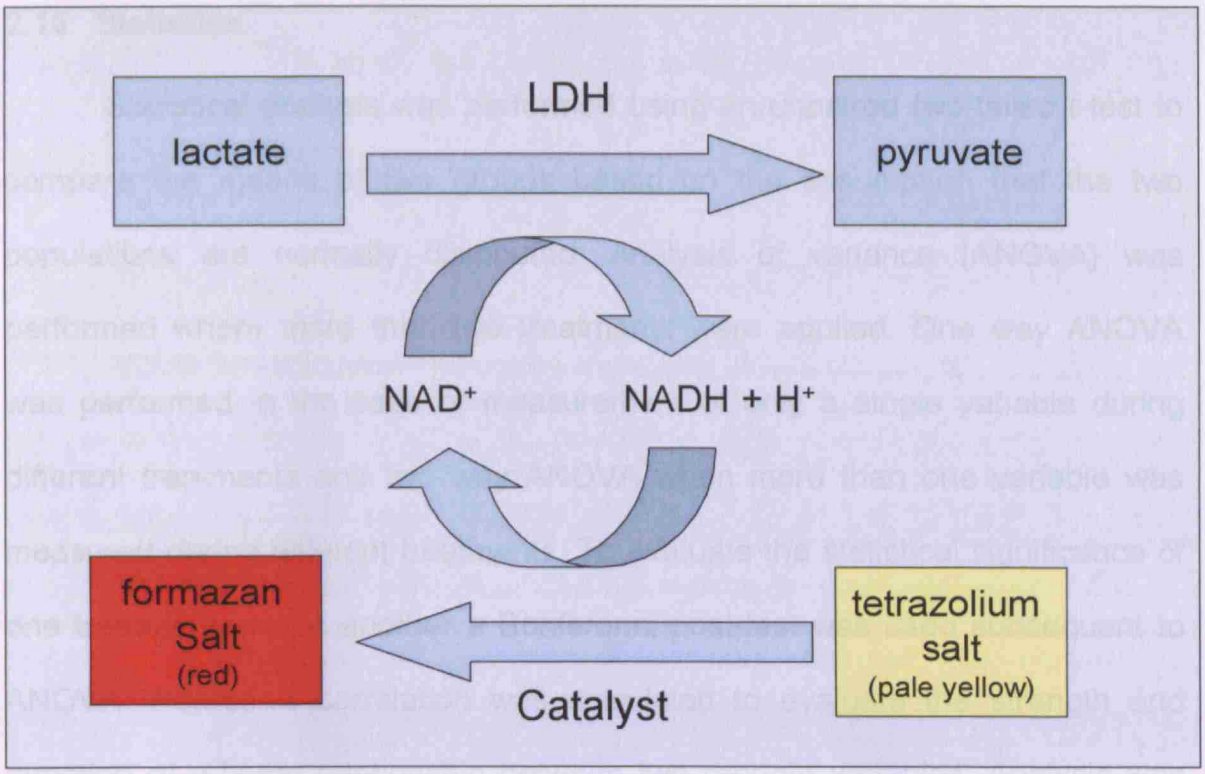


Figure 2-11. Principle behind lactate dehydrogenase colorimetric assay

An increase in the number of dead or plasma-membrane damaged cells results in an increase in the LDH enzyme activity in the culture supernatant. There was a direct correlation between the amount of enzyme activity and the amount of formazan produced during a limited time period. Therefore, the development of colour in the assay was proportional to the number of lysed cells. The water soluble dye formazan was detected spectrophotometrically at 490 nm using a Versamax tunable microplate reader (Molecular Devices Ltd, Berkshire, UK).

2.16 Statistics

Statistical analysis was performed using an unpaired two-tailed t-test to compare the means of two groups based on the assumption that the two populations are normally distributed. Analysis of variance (ANOVA) was performed where more than two treatments were applied. One way ANOVA was performed in the case of measurement of only a single variable during different treatments and two way ANOVA when more than one variable was measured during different treatments. To evaluate the statistical significance of one treatment versus another a Bonferonni post-test was used subsequent to ANOVA. Pearson's correlation was calculated to evaluate the strength and direction of a linear relationship between two random variables. Analysis was performed with Prism 4 (Graphpad Software).

Chapter 3. Effect of NO and haemoglobin interaction on endothelial haem oxygenase induction

3.1 Introduction

NO is an essential biological molecule and many of its physiological effects are mediated through its interaction with haem-containing proteins (Davis et al., 2001). In particular, the binding of NO to haemoglobin has been implicated in various physiological and pathophysiological processes, and can result in the generation of nitrosyl-haemoglobin (HbNO) or haem-nitrosyl (H-NO) complexes (Davies et al., 2005). Significantly, the enzyme HO-1 has been shown to be potently induced by both haemoglobin and NO molecules (Balla et al., 1993);(Motterlini et al., 1996a) and the interaction of haemin with NO has been shown to act synergistically in inducing endothelial HO-1 (Foresti et al., 2003), suggesting a possible role for HO-1 in pathological conditions characterised by increased haemolysis or NO production.

As both NO and haemoglobin are strong inducers of HO-1, we aimed in the present chapter to examine the effects of co-incubation of different haemoglobins with NO donors on endothelial HO-1 expression and haem oxygenase activity and attempt to identify potential mechanisms mediating this effect.

3.2 Objectives

- To investigate the effect of co-incubation of various haemoglobins with NO donors on the induction of endothelial haem oxygenase activity and HO-1 protein production

- To explore the role of the haem-nitrosyl (H-NO) complex in the induction of endothelial HO-1 activity, HO-1 expression and BR production

- To examine the effect of co-incubation of various haemoglobins with hydroxyurea on the induction of endothelial haem oxygenase activity and HO-1 expression

- To investigate whether haemoglobins modulate the release of NO by NO donors in culture media

3.3 Experimental protocol

The detailed methods used in the experiments described in this section can be found in the Materials and Methods chapter. Stock solutions of haemoglobins (methaemoglobin, HbA_0, HbS, HbF) and H-NO were prepared in PBS (see section 2.2 for H-NO preparation). Stock solutions (10 mM) of the NO donors S-Nitrosoglutathione (GSNO) and Angeli's salt (AS) were prepared in 0.01 M NaOH. Hydroxyurea stock solutions (10 mM) were prepared in distilled water. Haemin (1 mM) was prepared by dissolving it in 0.1 M NaOH and then adding 0.01 M phosphate buffer at pH 7.4. Haemoglobins were stored at -80°C, whereas H-NO, haemin, hydroxyurea and NO donor stock solutions were prepared fresh for each experiment. Incubations were carried out in the dark due to the light sensitive nature of the reagents.

3.3.1 Incubation of cells with reagents

To investigate the effect of co-incubation of haemoglobins and NO donors on endothelial haem oxygenase activity and HO-1 expression, three haemoglobins were used: 1) HbA_0, the form normally present in the adult red blood cell; 2) methaemoglobin, the oxidized form of haemoglobin; and 3) HbS, the mutated form of haemoglobin found in SCD. Cells were exposed to 15 μM HbA_0, 15 μM HbS or 1 μM methaemoglobin for 6 or 18 h in the presence or absence of the NO donor GSNO (250 or 500 μM) or the nitroxyl (NO^-) generator AS (250 μM). A lower concentration of methaemoglobin in comparison with HbA_0 and HbS was employed because preliminary experiments showed that 15 μM methaemoglobin was toxic to cells. Further experiments were performed to investigate the influence of a haem-nitrosyl complex on haem oxygenase activity, HO-1 expression and BR production. Endothelial cells were incubated

with either H-NO (15 μM), haemin (15 μM) or co-incubation of haemin (15 μM) and AS (250 μM) for 6 or 24 h. Haem oxygenase activity was assessed after 6 h, HO-1 expression was determined after 6 and 24 h and BR release in culture medium was measured after 24 h. To investigate the effect of hydroxyurea (the drug used in the treatment of SCD which may have NO releasing properties) on endothelial haem oxygenase activity, cells were incubated with 50 μM, 100 μM or 300 μM hydroxyurea for 6 h. Since hydroxyurea may possess NO donor characteristics, a potential interaction of the drug with haem in affecting haem oxygenase was explored by incubating endothelial cells with 15 μM haemin in the presence or absence of 300 μM hydroxyurea for 6 h. In additional experiments, the effect of co-incubation of hydroxyurea and haemoglobins on endothelial haem oxygenase activity and HO-1 expression was examined. HbF was investigated along with the three original haemoglobins utilized, since it is the form of haemoglobin produced following treatment of SCD patients with hydroxyurea. Therefore, endothelial cells were exposed to 15 μM HbA_0, HbS, HbF or 1 μM methaemoglobin for 6 or 18 h in the presence or absence of 300 μM hydroxyurea.

3.3.2 Haem oxygenase activity assay

Haem oxygenase activity was determined in endothelial cells at different times after treatment as described in section 2.7 Briefly, harvested cells were subjected to three cycles of freeze-thawing before addition to a reaction mixture containing NADPH, rat liver cytosol as a source of BVR, and the substrate haemin. The reaction was conducted at 37°C in the dark for 1 h, terminated by the addition of 1 ml chloroform, and the extracted BR was calculated by the difference in absorbance between 464 and 530 nm (ε = 40 $mM^{-1}cm^{-1}$).

3.3.3 Western-blot techniques for detection of HO-1 protein expression

Samples of endothelial cells also underwent Western immunoblot analysis as described in section 2.9. Briefly, an equal amount of proteins (30 μg) for each sample was separated by SDS-polyacrylamide gel electrophoresis, transferred overnight to nitrocellulose membranes, and the non-specific binding of antibodies was blocked with 3% non-fat dried milk in PBS. Membranes were then probed with a polyclonal rabbit anti-HO-1 antibody (Stressgen, Victoria, Canada) (1:1,000 dilution in Tris-buffered saline, pH 7.4). After three washes with PBS containing 0.05% (v/v) Tween 20, blots were visualized using an amplified alkaline phosphatase kit from Sigma (Extra-3A) and subjected to densitometric analysis. To ensure equal loading, cell samples also underwent Western-blot analysis for the house-keeping gene β-actin.

3.3.4 Determination of bilirubin in culture medium

For BR measurement cells were grown in 35 mm Petri dishes as described in section 2.11. At the end of the incubation, 0.5 ml of culture supernatant was added to 250 mg of $BaCl_2$ and vortex-mixed thoroughly as already described. 0.75 ml of benzene was then added to the mixture and tubes were vigorously vortex-mixed again. The benzene phase containing the extracted BR was separated from the aqueous phase by centrifugation at 13000 g for 30 min. BR was measured spectrophotometrically as a difference in absorbance between 450 and 600 nm (ε = 27.3 $mM^{-1} \cdot cm^{-1}$) and was expressed as nM/mg protein.

3.3.5 NO release measurement

The NO electrode was used to assess the effect of co-incubation of haemoglobins on NO release by NO donors. Once the NO electrode had equilibrated in media, additions of NO donors (250 μM AS, 250 μM GSNO, 300 μM hydroxyurea) in the presence or absence of haemoglobins (15 μM HbA_0, 15 μM HbS, 1 μM methaemoglobin) were made and the release of NO was measured over time (the length of measurement was dependent on the rate of release of NO in solution). Control experiments were also performed by adding either haemoglobins or NO donors alone to the NO electrode. All experiments were carried out under red light due to light-sensitive nature of the reagents.

3.4 Results

3.4.1 Both NO^+ (GSNO) and NO^- (AS) donors synergize with haemoglobin to increase endothelial haem oxygenase activity and up-regulate HO-1 expression

NO has been shown to strongly enhance the induction of HO-1 by haemin (Foresti et al., 2003);(Motterlini et al., 1996a; Naughton et al., 2002b) and it was investigated here whether similar effects could be observed with haemoglobin. Three separate haemoglobins were investigated because of their different behaviour in terms of haem release. In particular, HbA_0, which is stabilized in the ferrous form, becomes susceptible to oxidation once incubated with cells in aerobic conditions. A correlation exists between the rate of autoxidation of HbA_0 (i.e. rate of formation of methaemoglobin) and the increase in endothelial haem oxygenase activity. This can be attributed to the relative ease with which methaemoglobin can lose its haem moiety which then acts as an inducer for HO-1. In preliminary experiments, we observed that endothelial cells tolerate exposure to 15 μM HbA_0 for 6 or 18 h but are severely damaged in the presence of 15 μM methaemoglobin, thereby forcing us to lower the concentration of methaemoglobin to 1 μM. The mutated HbS was also tested because of its role in SCD and its intrinsic property to autoxidize and release haem faster.

HbA_0, HbS and methaemoglobin caused induction of endothelial HO-1 and a consequent increase in haem oxygenase activity (Figure 3-1, Figure 3-2 and Figure 3-3,) to differing levels. This effect was particularly evident after 18 h incubation, indicating that the time-dependent oxidation of haemoglobins and the subsequent release of haem from haemoglobin are important factors

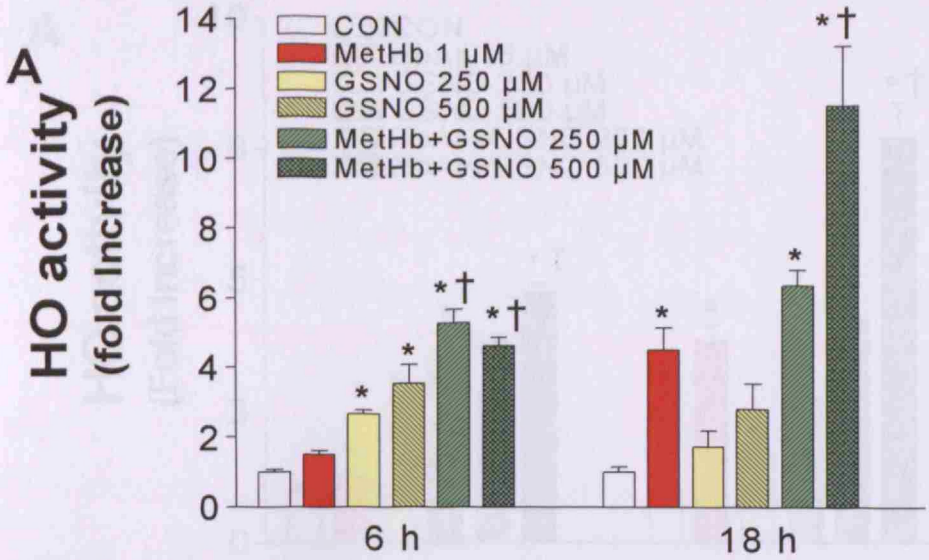
influencing HO-1 up-regulation. It was also observed that methaemoglobin produced greater endothelial haem oxygenase activity compared with HbA_0 (note that methaemoglobin was used at 1 μM vs. 15 μM HbA_0). However, when compared with the same concentration of HbS, HbA_0 produced a higher haem oxygenase activity. Therefore, the potency of the three haemoglobins in increasing haem oxygenase activity in endothelial cells could be summarized as follows: methaemoglobin > HbA_0 > HbS.

Incubation of endothelial cells with NO donors alone was also shown to induce haem oxygenase activity. For example, incubation with GSNO (Figure 3-1, Figure 3-2 and Figure 3-3) increased haem oxygenase activity in a concentration-dependent manner, with 500 μM GSNO showing greater haem oxygenase activity than 250 μM GSNO. In addition, endothelial cells treated with GSNO alone showed greater haem oxygenase induction at 6 h compared with 18 h, reflecting the fact the half-life of GSNO is less than 1 h, after which there is a reduction in NO release thus likely affecting the extent of haem oxygenase induction. The observed pattern of NO-mediated endothelial haem oxygenase induction was also seen with the AS (Figure 3-4), which has a half-life of only a few min. Specifically, AS-treated endothelial cells showed enhanced haem oxygenase activity at both 6 h and 18 h, with the higher of haem oxygenase levels at 6 h reflecting the pattern of NO^- release from AS. Therefore, treatment with GSNO and AS, independently from the NO species produced, resulted in enhanced haem oxygenase activity in endothelial cells.

Consistent with our previous findings using free haemin, NO in any oxidation state significantly enhanced the induction of HO-1 elicited by all haemoglobins at 6 h and 18 h (Figure 3-1, Figure 3-2, Figure 3-3 and Figure

3-4). The pattern of NO/haemoglobin mediated haem oxygenase induction was similar to that seen with cells treated with haemoglobins alone. Specifically, endothelial cells incubated with 1 μM methaemoglobin in the presence of NO donors showed greater haem oxygenase induction compared with cells exposed to 15 μM HbA_0 and NO donors. In addition, cells treated with HbS and NO donors exhibited the lowest haem oxygenase activity. After 18 h, GSNO continued to markedly amplify the haemoglobin-mediated increase in haem oxygenase activity, while the effect of AS was less pronounced. This is probably due again to the short half-life of AS (2-3 min) in physiological buffer, as opposed to the long-lasting NO releasing properties of GSNO.

The ability of NO to enhance haemoglobin-mediated haem oxygenase activity was also reflected in HO-1 protein production. Western blot analysis (Figure 3-1, Figure 3-2 and Figure 3-3), showed that induction of HO-1 protein was enhanced by incubation with methaemoglobin, HbA_0 or HbS alone, with 18 h showing greater HO-1 protein production than 6 h, thus reflecting the time-dependent release of haem. Incubation of cells with GSNO alone also produced enhanced HO-1 protein levels, with a higher level of protein detected at 18 h. Co-incubation of GSNO with all three haemoglobins showed a greater HO-1 protein production than that seen with either haemoglobin alone or GSNO alone.



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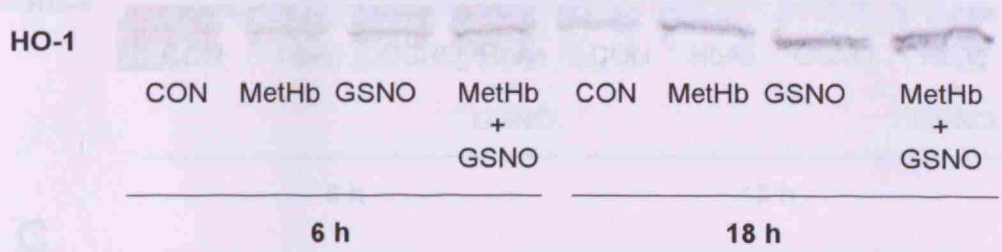
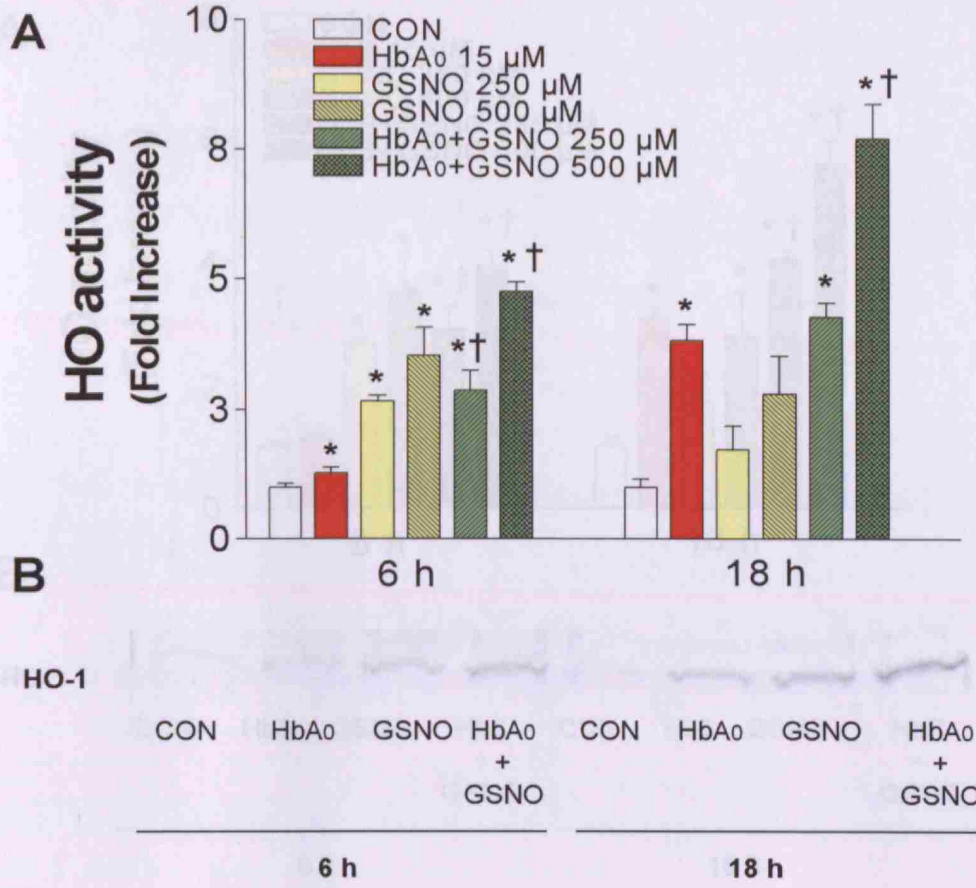


C

Densitometry (normalised HO-1/β-actin)				
Time	CON	MetHb (1 µM)	GSNO (500 µM)	MetHb+ GSNO
6 h	0.60±0.5	1.42±1.2	2.82±2.1	4.43±3.8
18 h	0.89±1.3	3.59±1.7	9.01±3.7	9.4±3.8

Figure 3-1. GSNO synergizes with methaemoglobin to up-regulate endothelial haem oxygenase activity and HO-1 expression

(A) Endothelial cells were exposed for 6 or 18 h to 1 µM methaemoglobin (MetHb) in the presence or absence of 250 or 500 µM S-nitrosogluthathione (GSNO). Cells were also exposed to GSNO or medium alone (CON). Haem oxygenase activity was measured as described in Materials and Methods. (B) The expression of HO-1 protein was determined by Western blot technique in cells incubated as above. The image is representative of three independent experiments and table C reports the mean arbitrary units of the results obtained by densitometric analysis of the bands (densitometry normalised to β-actin). Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. CON; † $P<0.05$ vs. MetHb alone.

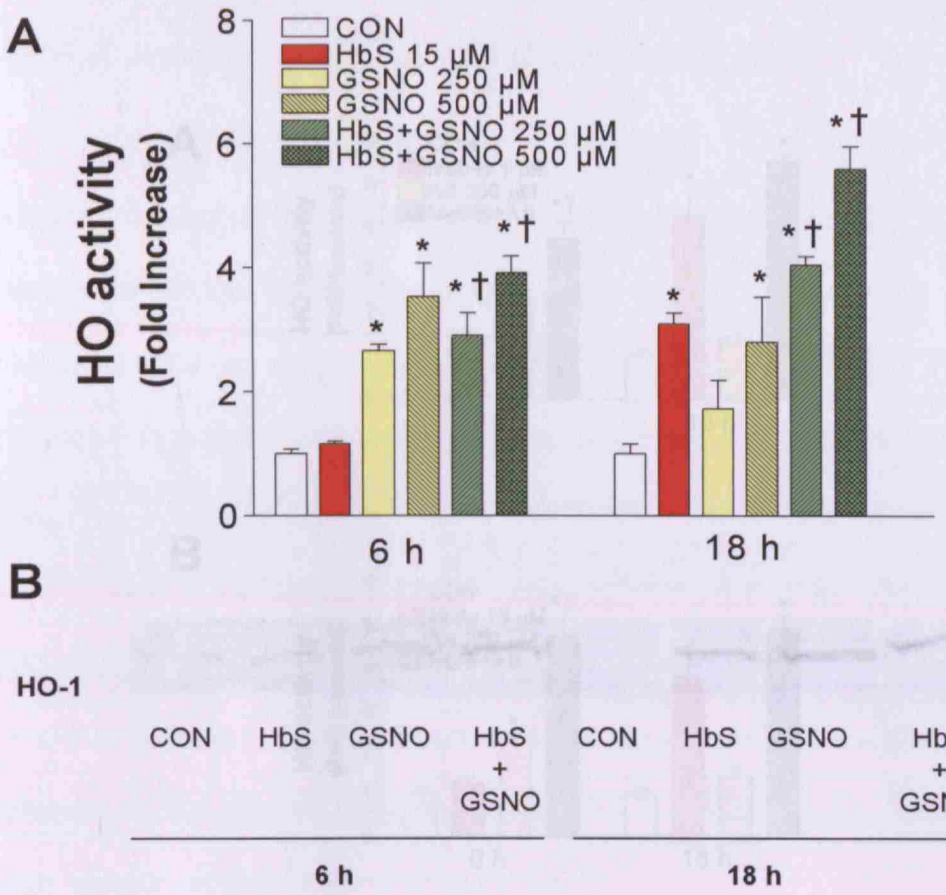


C

Densitometry (normalised HO-1/β-actin)				
Time	CON	HbA_0 (15 µM)	GSNO (500 µM)	HbA_0 + GSNO
6 h	0.92±0.7	1.78±1.1	4.26±1.5	8.10±4.5
18 h	0.66±0.5	4.33±3.0	7.0±5.2	15.0±6.6

Figure 3-2. GSNO synergizes with HbA_0 to up-regulate endothelial haem oxygenase activity and HO-1 expression

(A) Endothelial cells were exposed for 6 or 18 h to 15 µM human haemoglobin (HbA_0) in the presence or absence of 250 or 500 µM S-nitrosogluthathione (GSNO). Cells were also exposed to GSNO or medium alone (CON). Haem oxygenase (HO) activity was measured as described in Materials and Methods. (B) The expression of HO-1 protein was determined by Western blot technique in cells incubated as above. The image is representative of three independent experiments and table C reports the mean arbitrary units of the results obtained by densitometric analysis of the bands (densitometry normalised to β-actin). Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. CON; † $P<0.05$ vs. HbA_0 alone.



C

Densitometry (normalised HO-1/β-actin)				
Time	CON	HbS (15 µM)	GSNO (500 µM)	HbS + GSNO
6 h	0.09±0.1	0.57±0.2	1.03±0.6	3.38±2.5
18 h	0.23±0.2	4.15±2.1	3.79±3.1	8.60±4.7

Figure 3-3. GSNO synergizes with HbS to up-regulate endothelial haem oxygenase activity and HO-1 expression

(A) Endothelial cells were exposed for 6 or 18 h to 15 µM sickle cell haemoglobin (HbS) in the presence or absence of 250 or 500 µM S-nitrosogluthathione (GSNO). Cells were also exposed to GSNO or medium alone (CON). Haem oxygenase (HO) activity was measured as described in Materials and Methods. (B) The expression of HO-1 protein was determined by Western blot technique in cells incubated as above. The image is representative of three independent experiments and the table C reports the mean arbitrary units of the results obtained by densitometric analysis of the bands (densitometry normalised to β-actin). Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. CON; † $P<0.05$ vs. HbS alone.

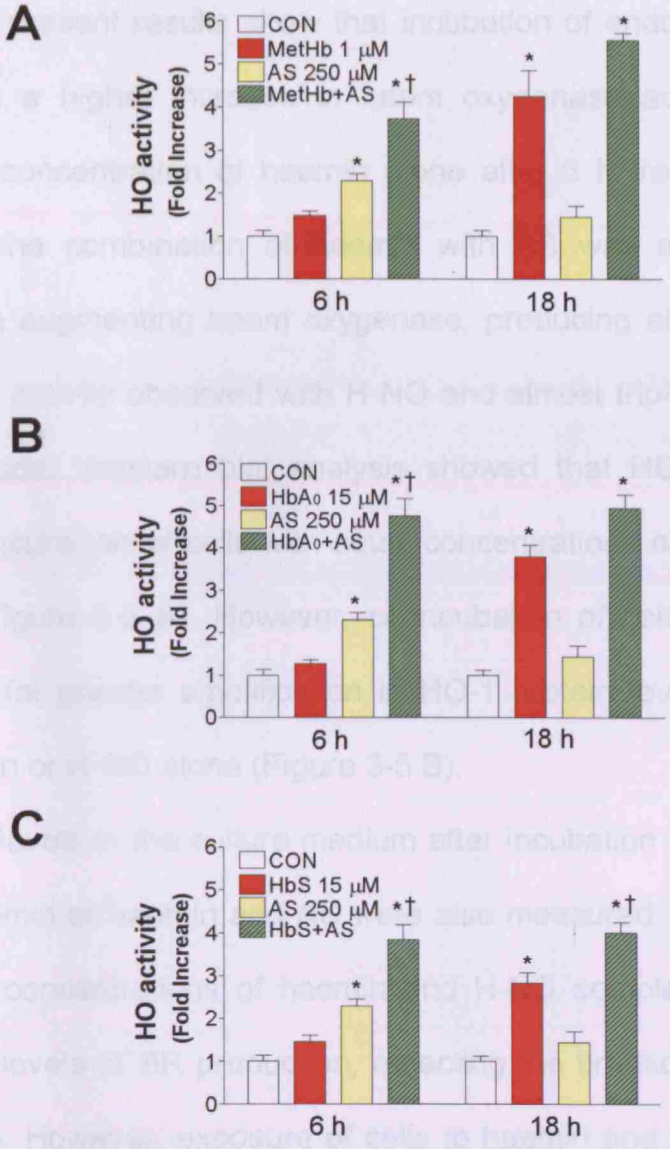


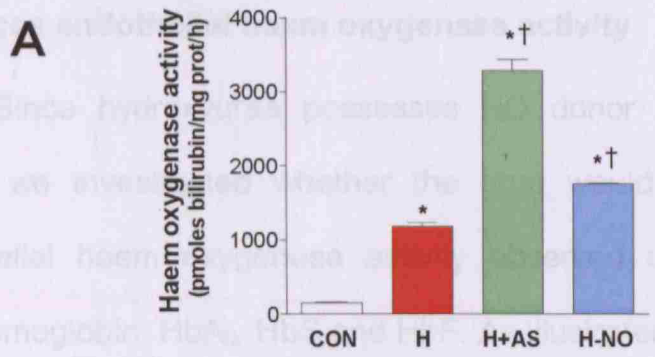
Figure 3-4. A nitroxyl generator synergizes with different haemoglobins to up-regulate endothelial haem oxygenase activity

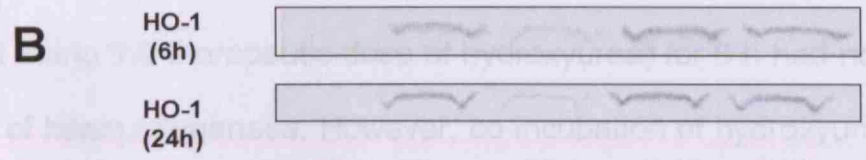
(A) Endothelial cells were exposed for 6 or 18 h to 1 μM methaemoglobin (MetHb) in the presence or absence of 250 μM Angeli's salt (AS, a nitroxyl generator) for the haem oxygenase (HO) activity assay. Cells were also exposed to AS or medium alone (CON). Similar experiments were conducted to measure haem oxygenase activity with HbA_0 (B) and HbS (C). For the haem oxygenase activity assay, bars represent the mean±SEM of 4-6 independent experiments per group. *P<0.05 vs. CON; †P<0.05 vs. each different haemoglobin alone.

3.4.2 Comparison between haem-nitrosyl and haemin on the stimulation of haem oxygenase activity and bilirubin production

The present results show that incubation of endothelial cells with H-NO resulted in a higher increase in haem oxygenase activity compared to an equimolar concentration of haemin alone after 6 h treatment (Figure 3-5 A). However, the combination of haemin with AS was shown to be the most powerful in augmenting haem oxygenase, producing almost double the haem oxygenase activity observed with H-NO and almost triple the activity seen with haemin alone. Western blot analysis showed that HO-1 levels were similar following incubation of cells with equal concentrations of haemin or H-NO for 6 or 24 h (Figure 3-5 B). However, co-incubation of cells with haemin and AS showed a far greater amplification in HO-1 protein levels than that observed with haemin or H-NO alone (Figure 3-5 B).

BR levels in the culture medium after incubation of endothelial cells with H-NO, haemin or haemin and AS were also measured. Incubation of cells with equimolar concentrations of haemin and H-NO complex showed comparable enhanced levels of BR production, reflecting the breakdown of haem by haem oxygenase. However, exposure of cells to haemin and AS together resulted in much higher BR levels in the culture medium than seen with either haemin or H-NO alone (Figure 3-5 C).





Densitometry (normalised HO-1/β-actin)					
Time (h)	CON	Haemin (15 μ M)	AS (250 μ M)	Haemin/AS	H-NO (15 μ M)
6 h	0.83±1.0	18.3±6.3	8.33±3.9	21.17±5.5	22.44±6.5
24 h	1.77±1.2	24.8±3.9	8.67±4.1	41.4±16.9	24.62±7.5

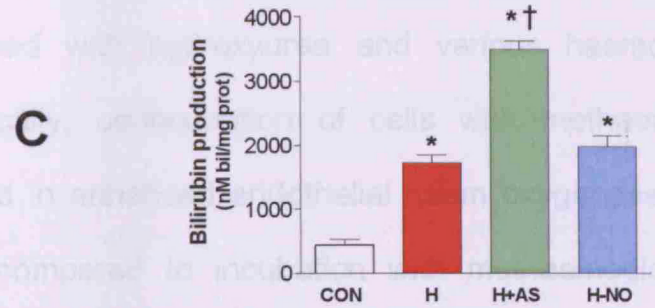


Figure 3-5. Effect of haem-nitrosyl on haem oxygenase activity, HO-1 expression and bilirubin production

Cells were incubated with medium alone (CON), haemin (H, 15 μM), haemin in the presence of 250 μM AS (H+AS) or a haem-nitrosyl complex (H-NO, 15 μM) for 6 or 24 h. At the end of the incubation, samples were collected for (A) the haem oxygenase activity assay (6 h) or (B) for determination of HO-1 protein levels (6 and 24 h). The Western blot is representative of three independent experiments; the table reports the mean arbitrary units of the results obtained by densitometric analysis of the bands (densitometry normalised to β-actin). (C) Bilirubin released into the culture supernatant was measured 24 h following exposure of cells with medium alone (CON), 15 μM haemin (H), haemin in the presence of 250 μM AS (H+AS) or a haem-nitrosyl complex (H-NO, 15 μM). Data represent the mean±SEM of 5-6 independent experiments per group. * $P<0.05$ vs. CON; † $P<0.05$ vs. haemin alone.

3.4.3 Co-incubation of hydroxyurea with haemin or haemoglobins enhances endothelial haem oxygenase activity

Since hydroxyurea possesses NO donor properties (Gladwin et al., 2002), we investigated whether the drug would influence the increase in endothelial haem oxygenase activity observed upon exposure to haemin, methaemoglobin, HbA_0, HbS and HbF. As illustrated in Figure 3-6 A, incubation of endothelial cells with increasing concentrations of hydroxyurea (0-300 μM, 300 μM being the therapeutic dose of hydroxyurea) for 6 h had no effect on the activity of haem oxygenase. However, co-incubation of hydroxyurea with 15 μM haemin resulted in significant enhanced induction of endothelial haem oxygenase activity compared to incubation with hydroxyurea or haemin alone (Figure 3-6 B).

Comparable results were observed when endothelial cells were co-incubated with hydroxyurea and various haemoglobins for 6 and 18 h. Specifically, co-incubation of cells with methaemoglobin and hydroxyurea resulted in enhanced endothelial haem oxygenase activity at both 6 and 18h when compared to incubation with methaemoglobin alone (Figure 3-7 A). However, co-incubation of 15 μM HbA_0 or HbS with hydroxyurea only elicited enhanced haem oxygenase activity at 18 h when compared with exposure to haemoglobins alone, possibly reflecting the time dependent release of haem from these two haemoglobins (Figure 3-7 B, C). In contrast, co-incubation of hydroxyurea with HbF showed increased haem oxygenase activity only at 6 h when compared to HbF incubation, suggesting a difference in the haem releasing properties of this particular haemoglobin.

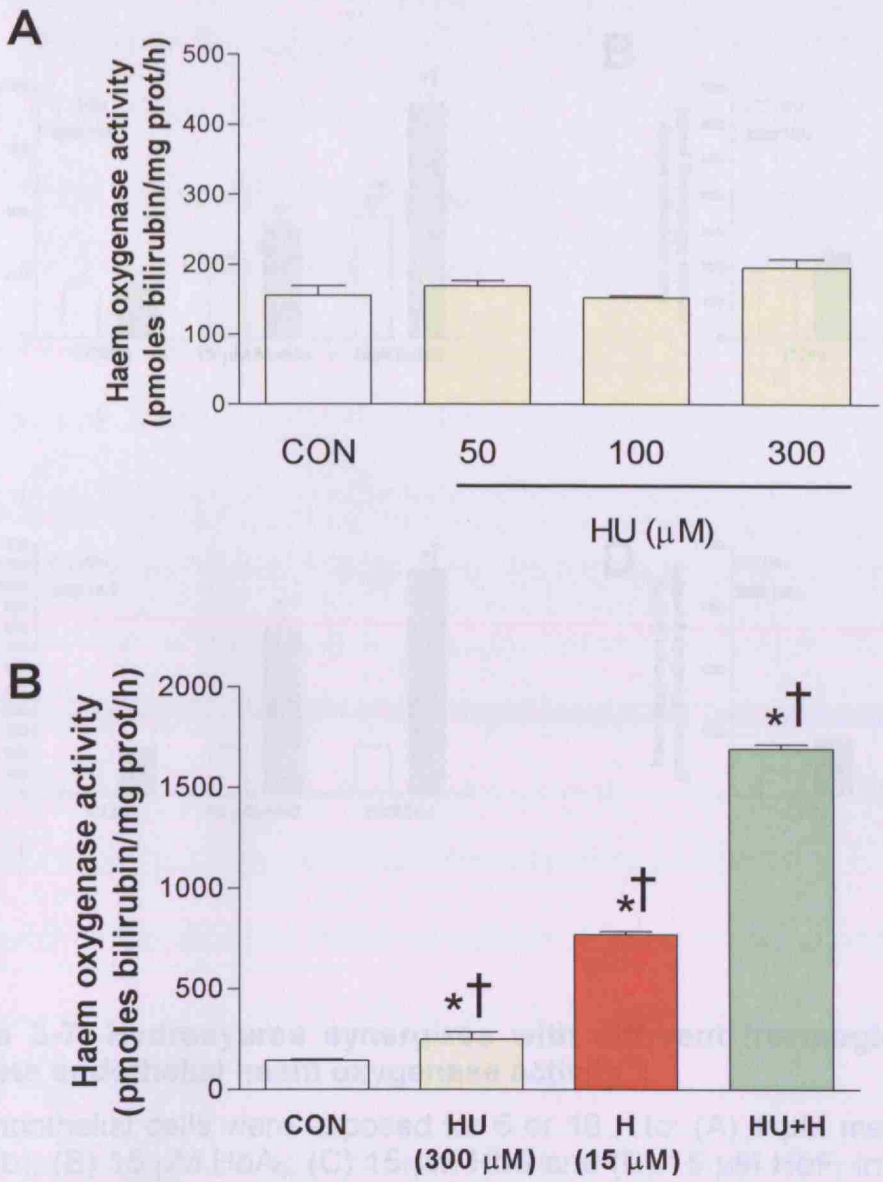


Figure 3-6. Effect of hydroxyurea alone or in combination with haem on haem oxygenase activity

Endothelial cells were incubated with (A) 50 μM, 100 μM or 300 μM hydroxyurea (HU) for 6 h and (B) 15 μM haemin (H) in the presence (HU+H) or absence of 300 μM HU for 6 h. Cells were also exposed to HU or medium alone. At the end of the experiment, samples were collected for the haem oxygenase activity. Data represent the mean±SEM of 5-6 independent experiments per group. *P<0.05 vs. CON; † P<0.05 vs. haemin alone.

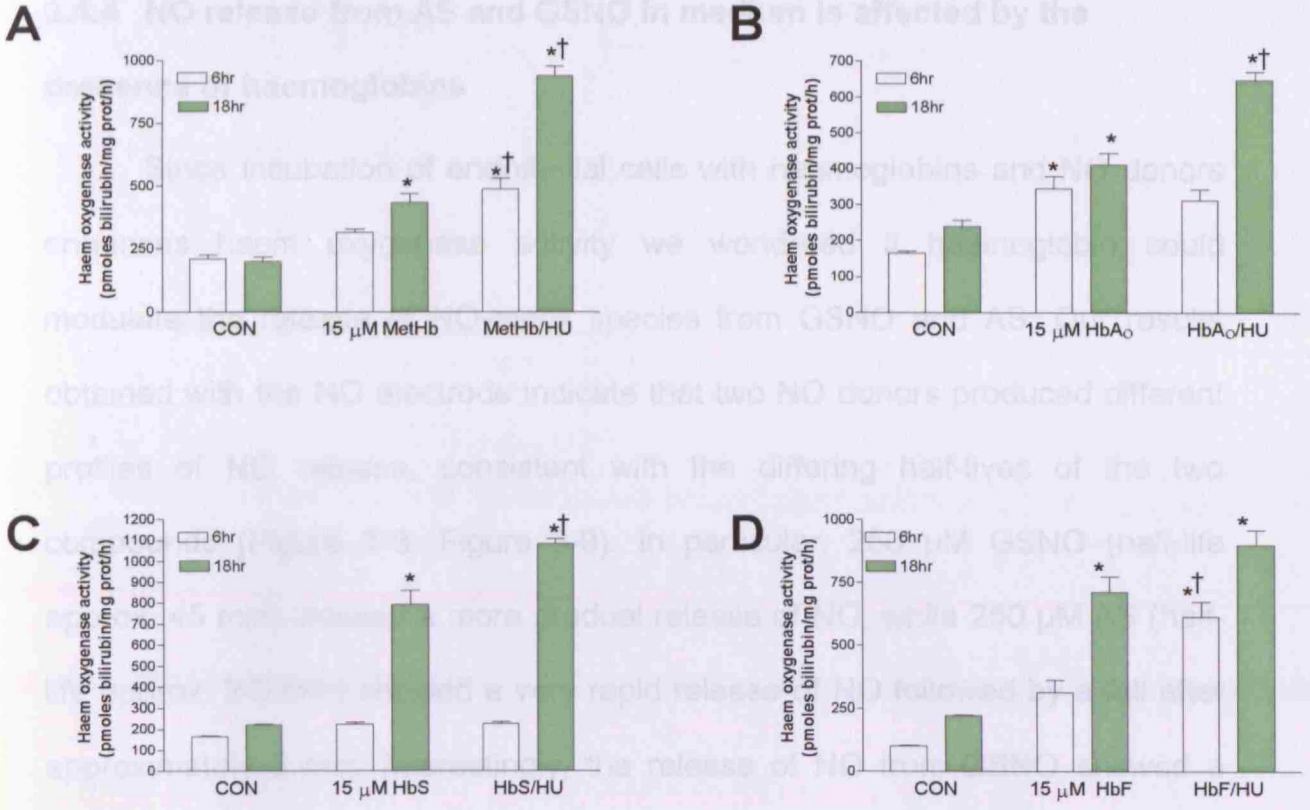


Figure 3-7. Hydroxyurea synergizes with different haemoglobins to up-regulate endothelial haem oxygenase activity

(A) Endothelial cells were exposed for 6 or 18 h to: (A) 1 μM methaemoglobin (MetHb); (B) 15 μM HbA_0; (C) 15 μM HbS and (D) 15 μM HbF, in the presence or absence of 300 μM hydroxyurea (HU). Cells were also exposed to medium alone (CON). Haem oxygenase activity assay was performed at the end of the experiment as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. CON; † $P<0.05$ vs. each different haemoglobin alone.

3.4.4 NO release from AS and GSNO in medium is affected by the presence of haemoglobins

Since incubation of endothelial cells with haemoglobins and NO donors enhances haem oxygenase activity we wondered if haemoglobin could modulate the release of NO-redox species from GSNO and AS. Our results obtained with the NO electrode indicate that two NO donors produced different profiles of NO release, consistent with the differing half-lives of the two compounds (Figure 3-8, Figure 3-9). In particular, 250 μM GSNO (half-life approx. 45 min) showed a more gradual release of NO, while 250 μM AS (half-life approx. 2-3 min) showed a very rapid release of NO followed by a fall after approximately 5 min. Interestingly, the release of NO from GSNO showed a marked reduction in the presence of 15 μM HbA_0 or HbS (Figure 3-8 B, C). The presence of the oxidised methaemoglobin (Figure 3-8 A) promoted a greater release of NO compared to GSNO alone. In contrast, all three haemoglobins prolonged the release of NO from AS, with methaemoglobin (Figure 3-9 A) producing the greatest NO release from the NO donor when compared to HbA_0 and HbS (Figure 3-9 B, C). Therefore, our data suggest that the oxidation state of the haemoglobin effects the releasing properties of GSNO and AS.

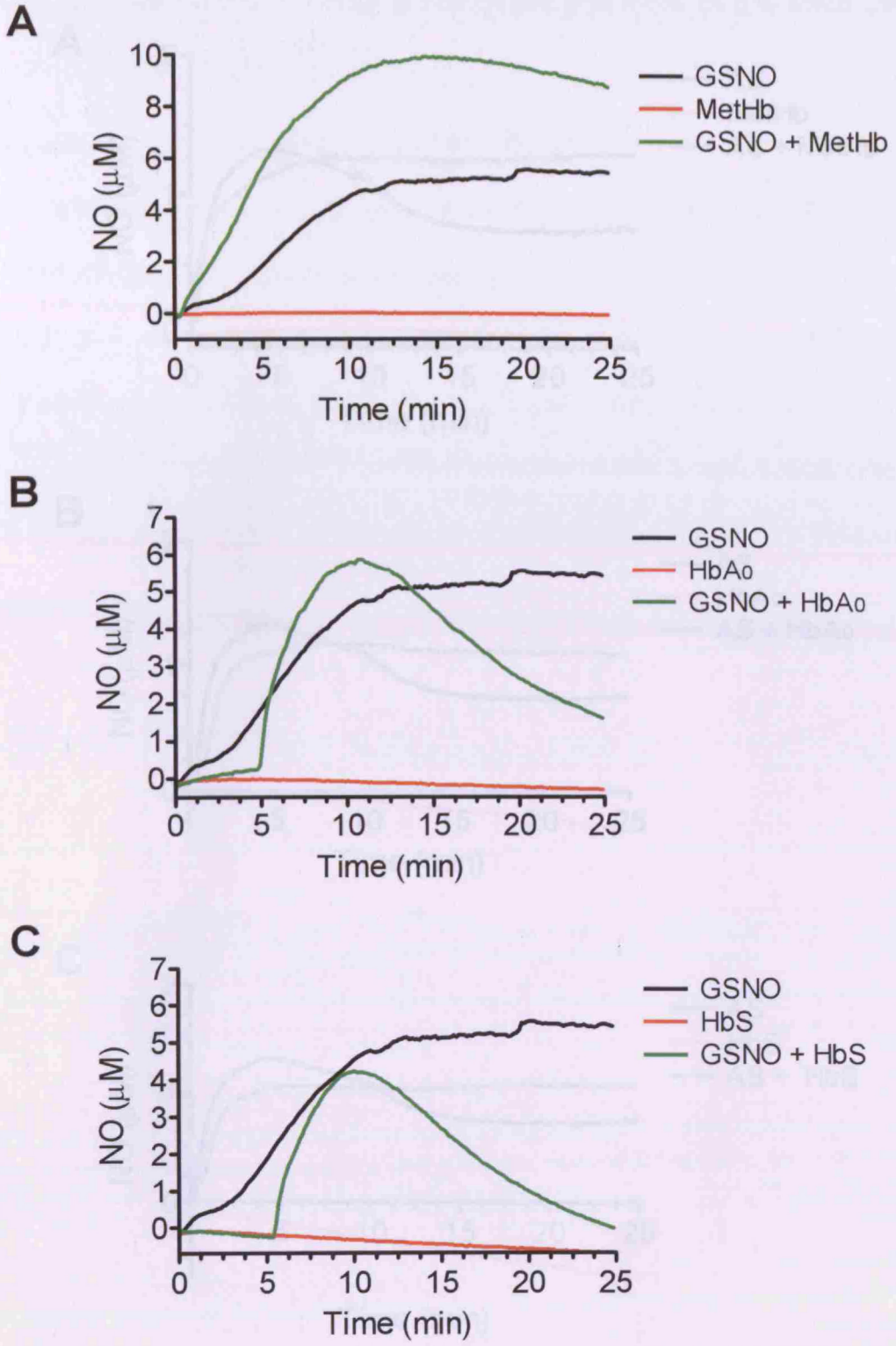


Figure 3-8. Release of NO from GSNO in the presence of haemoglobins

An NO electrode was used to detect the amount of NO released from GSNO (250 μM) into medium in the presence of (A) 1 μM methaemoglobin (MetHb) (B) 15 μM HbA_0, and (C) 15 μM HbS. Representative traces of NO detection over time are shown.

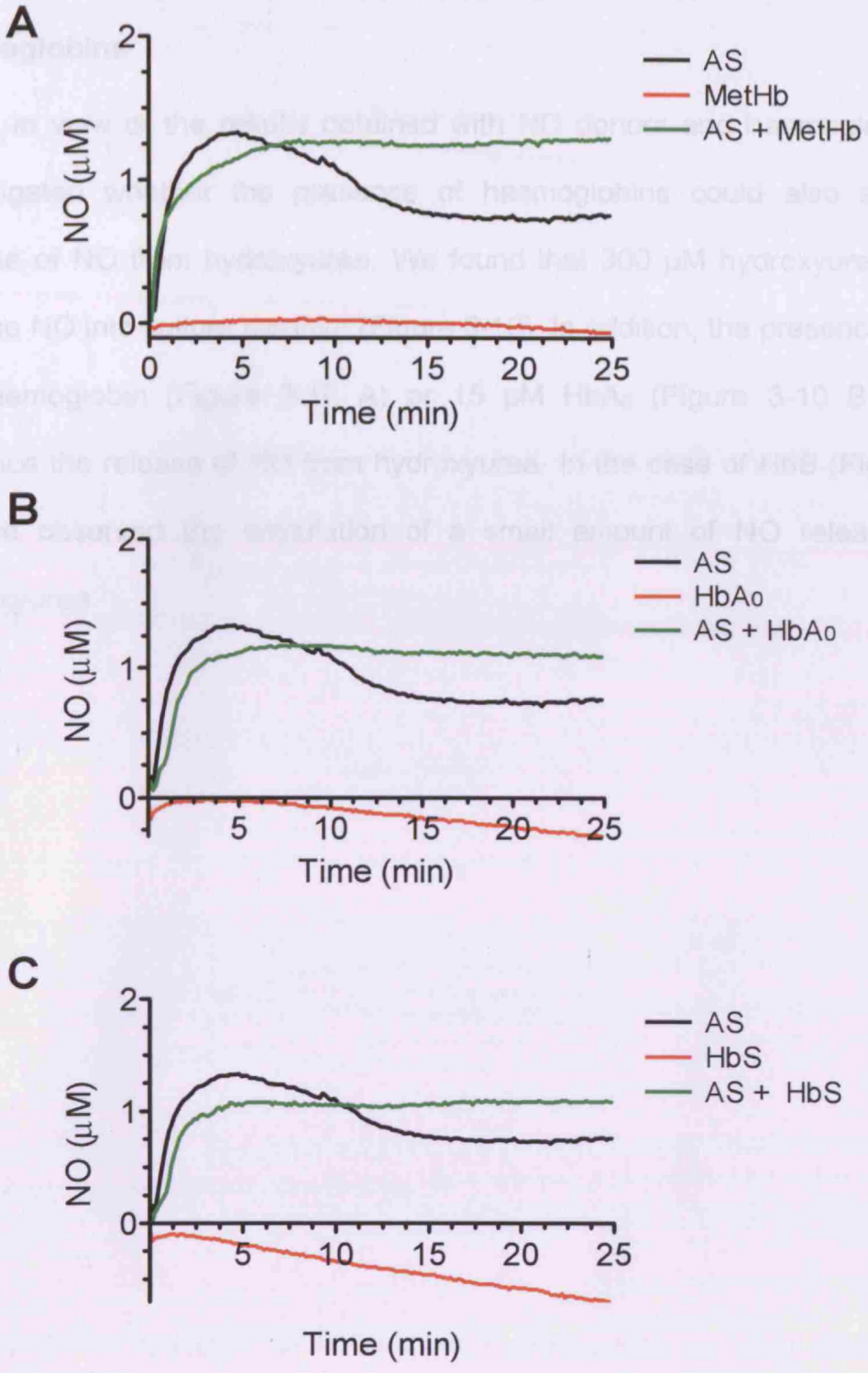


Figure 3-9. Release of NO from AS in the presence of haemoglobins

An NO electrode was used to detect the amount of NO released from AS (250 μM) into medium in the presence of (A) 1 μM methaemoglobin (MetHb) (B) 15 μM HbA_0, and (C) 15 μM HbS. Representative traces of NO detection over time are shown.

3.4.5 NO release from hydroxyurea in the presence or absence of haemoglobins

In view of the results obtained with NO donors and haemoglobins, we investigated whether the presence of haemoglobins could also affect the release of NO from hydroxyurea. We found that 300 μM hydroxyurea did not release NO into culture medium (Figure 3-10). In addition, the presence of 1 μM methaemoglobin (Figure 3-10 A) or 15 μM HbA_0 (Figure 3-10 B) did not enhance the release of NO from hydroxyurea. In the case of HbS (Figure 3-10 C), we observed the stimulation of a small amount of NO released from hydroxyurea.

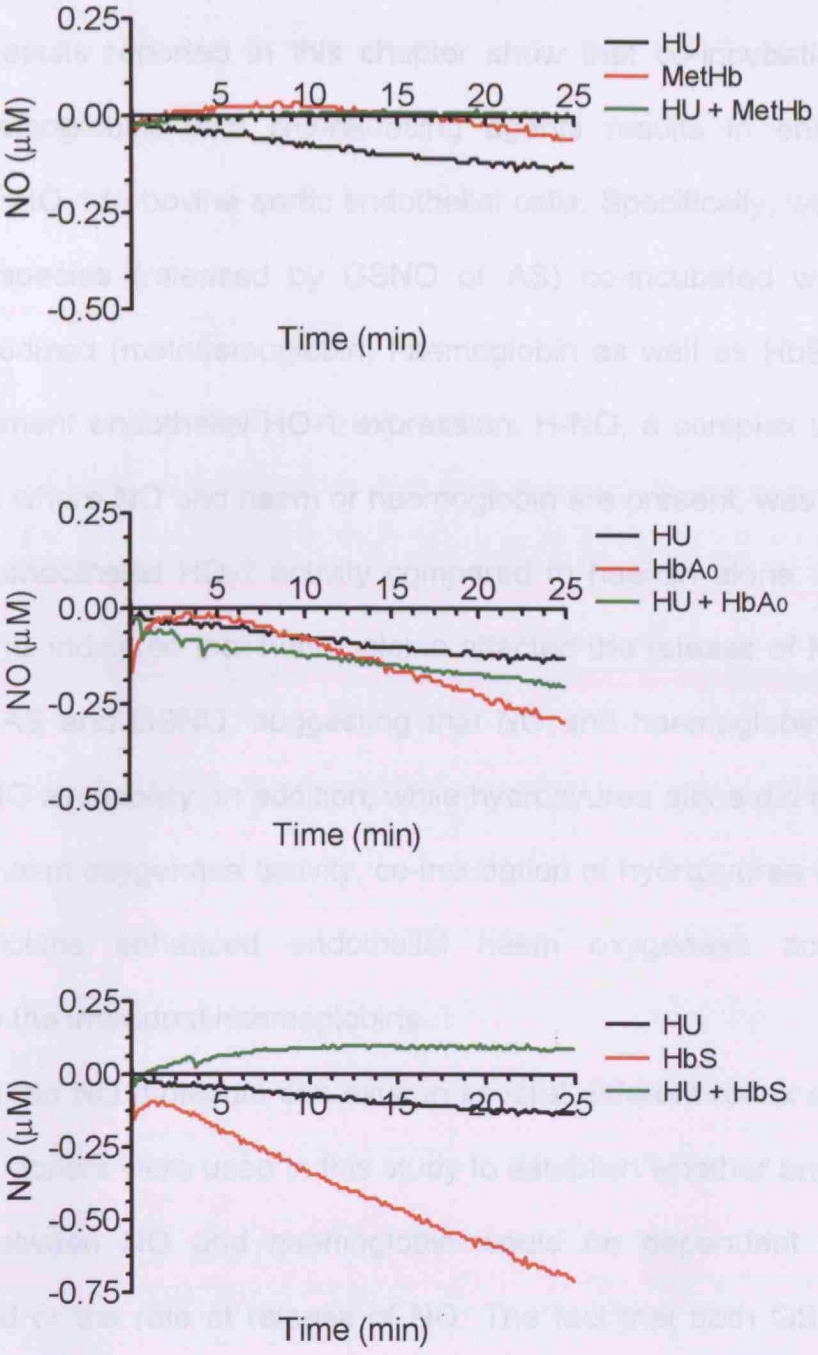


Figure 3-10. Release of NO from hydroxyurea in the presence of haemoglobins

An NO electrode was used to detect the amount of NO released from 300 μM hydroxyurea (HU) into medium in the presence of (A) 1 μM methaemoglobin (MetHb) (B) 15 μM HbA_0, and (C) 15 μM HbS. Representative traces of NO detection over time are shown.

3.5 Discussion

The results reported in this chapter show that co-incubation of three different haemoglobins with NO-releasing agents results in enhanced up-regulation of HO-1 in bovine aortic endothelial cells. Specifically, we found that NO-related species (released by GSNO or AS) co-incubated with reduced (HbA_0) or oxidized (methaemoglobin) haemoglobin as well as HbS from SCD strongly augment endothelial HO-1 expression. H-NO, a complex which forms in conditions where NO and haem or haemoglobin are present, was also shown to enhance endothelial HO-1 activity compared to haemin alone. NO release measurements indicated that haemoglobin affected the release of NO from the NO donors AS and GSNO, suggesting that NO and haemoglobin interaction may affect NO availability. In addition, while hydroxyurea alone did not enhance endothelial haem oxygenase activity, co-incubation of hydroxyurea with haemin or haemoglobins enhanced endothelial haem oxygenase activity when compared to the individual haemoglobins.

Since the NO molecule can exist in several different redox species, two different NO donors were used in this study to establish whether any synergism observed between NO and haemoglobin would be dependent on the NO species used or the rate of release of NO. The fact that both GSNO and AS produced an enhancement in haem oxygenase activity when co-incubated with haemoglobin indicates that the synergism observed was independent of NO species. However, important differences were also observed: 1) AS, which rapidly reacts with haemoglobin to form the ferrous nitrosyl adducts, produced the largest and most profound amplification at 6 h incubation; and 2) GSNO amplified haem oxygenase activity to a greater extent than AS after 18 h

incubation. These results suggest that the induction of HO-1 by NO-releasing agents correlates better with the rate of liberation of the NO redox form (i.e. the half-life of the NO donor rather) than the NO species generated from the compound , as already observed in previous reports (Foresti and Motterlini, 1999; Motterlini et al., 2002b).

It should be noted that incubation of endothelial cells with GSNO alone elicited contrasting results when haem oxygenase activity and protein levels were compared (Figure 3-1, Figure 3-2 and Figure 3-3). Specifically, GSNO enhanced haem oxygenase activity at both 6 and 18 h with the highest levels observed at 6 h. Conversely, GSNO stimulated higher HO-1 protein levels at 18 h than after 6 h. An explanation for these observations could be a potential inhibitory action of NO on haem oxygenase activity. Indeed, NO has been shown to attenuate the catalytic activity of both HO-1 and HO-2 isoforms of the enzyme in several studies (Ding et al., 1999; Wang et al., 2003).

Interestingly, the data obtained with the NO electrode showed that haemoglobin may enhance NO bioavailability of NO donors. This was especially evident for AS, and among the different haemoglobins methaemoglobin elicited the greatest NO release. A possible explanation of these results could be the binding of haem with NO species, resulting in a complex which may prolong the availability of NO in the medium. Therefore, the observation that methaemoglobin promoted the greatest NO release from NO donors could result from enhanced release of haem from the oxidised haemoglobin, when compared to HbA_0 an HbS, which could potentially interact with and extend the availability of NO. However, a different picture was seen with NO release by GSNO, where the presence of HbA_0 and HbS accelerated the removal of NO

from the medium, suggesting that NO had been scavenged by the haemoglobin. As in the case of AS, methaemoglobin elicited a large NO release from GSNO compared with GSNO alone, suggesting that the NO may have bound to free haem to form a stable complex. These observations may help to understand how NO donors enhanced the induction of HO-1 elicited by haemoglobin, although a number of different mechanisms explain this effect. For example, the enhanced release of NO from NO donors stimulated by haemoglobins could intensify HO-1 up-regulation. Furthermore, NO may form complexes with haemoglobin or haem, and these complexes *per se* may be more powerful in inducing HO-1.

It is important to note that many chemical transformations can occur in the presence of NO and haemoglobin. Both the NO^+ and NO^- donors used have been shown to undergo conversion to NO; for example, GSNO is converted to NO by copper (Singh et al., 1996) or haem (Spencer et al., 2000), while AS is oxidized to NO by reaction with oxygen (Han et al., 2002). However, despite the many fundamental reactions that may occur between the different states of haemoglobin and NO or H-NO, the final products appear to be the same in as much as the ferrous nitrosyl complex (NO-Hb) will be formed from reactions of NO^x donors with deoxy or methaemoglobin, while the reactions of oxyhaemoglobin with NO^x generally produce the oxidized methaemoglobin (Pawloski et al., 2001). Methaemoglobin will also slowly convert into NO-Hb in the presence of NO gas, which exhibits a long half-life in oxygenated solutions (Herold and Rock, 2003). Based on these notions, we postulated a key role for the ferrous nitrosyl haem in HO-1 induction elicited by co-incubation of haem or haemoglobins with NO donors.

Our findings sustain the idea that H-NO induces greater HO-1 expression than haem. However, the effect of H-NO was much less pronounced than that obtained with a simultaneous incubation of haemin plus AS, suggesting that formation of H-NO may not be the only mechanism involved in the synergism seen between haemoglobin and NO donors on HO-1 induction. Another potential mechanism is the enhanced release of NO by NO donors in the presence of haemoglobins, as highlighted by the NO electrode results.

The type of haemoglobin used appeared to be an important factor affecting the extent of HO-1 induction. Methaemoglobin, used at a concentration of only 1 μM due to its cytotoxic effects, stimulated levels of HO-1 induction comparable to 15 μM HbA_0 and HbS. In addition, 1 μM methaemoglobin incubated with NO donors resulted in a greater enhancement of haem oxygenase activity compared to that obtained with HbA_0 or HbS. These results suggest that oxidised haemoglobin is a better inducer of HO-1 and that NO-related species may synergize more efficiently with oxidized haemoglobin to induce HO-1. This point is corroborated by the observation that higher levels of HO-1 expression were measured after 18 h incubation compared to 6 h for both HbA_0 and HbS, suggesting that up-regulation of HO-1 relies on the time-dependent oxidation of haemoglobins, with subsequent conversion into methaemoglobin and increased release of haem. Since HbS is known to be more susceptible to haem loss (Reiter et al., 2002), it was surprising to observe that co-incubation of HbS with NO donors elicited a smaller amplification effect in comparison with HbA_0. Recent reports highlighting the impaired reactivity and processing of NO by the mutated HbS molecule may explain the observed results, as it has been postulated that abnormal binding of NO to sickle red

blood cells may be partly responsible for the abnormal vasodilatation seen in this disease state (Pawloski et al., 2005).

The increased haem oxygenase activity observed following co-incubation of haemin or haemoglobins with hydroxyurea is also an interesting finding. Although the main therapeutic benefit of hydroxyurea treatment in SCD is attributed to enhancement of HbF production and consequent reduction of unstable HbS (Gladwin et al., 2002), our findings indicate another potential therapeutic mechanism of action for the drug through up-regulation of haem oxygenase and the production of the protective breakdown products of haem. The variation in the magnitude of haem oxygenase induction between the different haemoglobins may again be explained by differences in the properties and haem release of the individual haemoglobins. Notably, hydroxyurea alone did not enhance endothelial haem oxygenase activity, but requires the presence of haemin or haemoglobin. If NO release by hydroxyurea is part of the mechanisms mediating haem oxygenase induction when the drug is incubated with haem or haemoglobins, it could be speculated that hydroxyurea may act by transferring NO to a haem or haemoglobin molecule (Jiang et al., 1997), thereby forming a complex responsible for up-regulation of haem oxygenase. Sustaining this idea are the findings that sickle cell patients receiving hydroxyurea treatment exhibit increased levels of nitrosylated haemoglobins (Gladwin et al., 2002). These patients were also found to have high plasma nitrite and nitrate levels, suggesting that an NO species was indeed being generated from hydroxyurea. We observed that HbS, but not the other haemoglobins tested, elicited a slight increase of NO release from hydroxyurea suggesting that the abnormal chemical structure of HbS has a role in promoting

the release of NO from hydroxyurea. However, there are many other components in the blood of sickle cell patients which may influence the release of NO from hydroxyurea and it would be difficult to reproduce the same pathophysiological scenario in our experiments. Interpreting our results from a clinical perspective, enhanced haem oxygenase activity could only take place under conditions of haemolysis, a characteristic of SCD (Bunn, 1997) where both hydroxyurea and haem/haemoglobin would be present. This may provide beneficial outcomes, as hydroxyurea would trigger HO-1 induction only when it was needed, i.e. during a vascular occlusive crises when red blood cells would sickle and release HbS. In addition, although the scavenging of NO by haemoglobins has often been considered as having detrimental effects on vascular homeostasis (Reiter et al., 2002), this same reaction may represent a protective mechanism in preventing the deleterious effects of excess haem observed in haemolytic condition such as SCD, since the interaction of NO and haem/haemoglobin leads to enhanced levels of the cytoprotective HO-1.

In conclusion, our results demonstrate a synergism between NO and haem/haemoglobin in up-regulating HO-1 in endothelial cells and resulting in increased production of haem metabolic products. The formation of a H-NO complex may be one of the possible mechanisms involved in this effect in conjunction with changes in the release of NO by NO donors in the presence of haemoglobin. Extending these findings to hydroxyurea, the observation reported in this chapter may have important implications for SCD and other haemolytic disorders.

Chapter 4. Effect of NO and haemoglobin interaction on endothelial haem uptake

4.1 Introduction

Despite haem being a vital molecule involved in many essential cellular functions, free haem is a highly toxic molecule and promotes oxidative stress (Ponka, 1999). Therefore, cells have evolved specific mechanisms to maintain free toxic haem or haemoglobin at low concentrations; among these are systems controlling the synthesis and degradation of haem by the enzymes ALA synthase and haem oxygenase, respectively. In addition, the binding of haem to specific protein carriers such as albumin, haemopexin or haptoglobin (Morgan et al., 1976);(Kristiansen et al., 2001) contributes to fine control of the potential toxicity of this porphyrin molecule.

Increased extracellular haem results in an increase in uptake of haem into cells and this may be important in the pathological states characterized by elevated haem levels such as endotoxic/haemorrhagic shock or SCD. For example, the incorporation of haem intracellularly, which may or may not be regulated by external factors, may participate in the resolution of these pathological states via HO-1 induction.

Our previous work investigating the influence of NO on haem-mediated modulation of endothelial HO-1 expression led us to some intriguing findings (Foresti et al., 2003), whereby co-incubation of endothelial cells with haemin and NO resulted in an increase in haem uptake and a subsequent magnification in endothelial HO-1 induction. We propose that similar reactions might take place when elevated levels of haem are associated with elevated levels of NO

in disease states and the results presented in the previous chapter also support a synergistic action of NO and haemoglobins in up-regulating HO-1. The aim of the present chapter was to examine the effect of NO donors and hydroxyurea on the uptake of haem promoted by incubation of endothelial cells with different haemoglobins.

4.2 Objectives

- To study the effect of co-incubation of various haemoglobins with NO donors on the uptake of haem by endothelial cells

- To investigate the effect of haem-nitrosyl complex (H-NO) on the uptake of haem by endothelial cells

- To investigate the effect of co-incubation of various haemoglobins with hydroxyurea on the uptake of haem by endothelial cells

- To explore the possible mechanism of action of NO on enhanced haem uptake

- To examine the effect of haem and NO incubation on various cell types

4.3 Experimental protocol

The specific methods used in the experiments described in the following section can be found in the Materials and Methods chapter. Stock solutions of haemoglobins (methaemoglobin, HbA_0, HbS) and H-NO were prepared in PBS and a stock solution of Angeli's salt (AS) was prepared in 0.01 M NaOH. Hydroxyurea stock solutions (10 mM) were prepared in distilled water. A stock solution of haemin (1 mM) was prepared by dissolving it in 0.1 M NaOH and then adding 0.01 M phosphate buffer at pH 7.4. Haemoglobins were be stored at -80°C, whereas H-NO, haemin, hydroxyurea and AS were prepared fresh for each experiment. Incubations were carried out in the dark due to the light sensitive nature of the reagents.

4.3.1 Incubation of cells with reagents

To determine the influence of NO or nitroxyl on endothelial haem uptake, endothelial cells were incubated with 15 μM haemin, 1 μM methaemoglobin, 15 μM HbA_0 or 15 μM HbS in the presence or absence of AS (250 μM) and haem content was measured after 2, 4 and 6 h. The effects of a pure haem-nitrosyl complex (15 μM) on haem uptake were also investigated. The influence of serum albumin on haem uptake was analyzed by incubating cells with haemin (15 μM) in serum-free medium or serum-free medium containing bovine serum albumin (5 or 30 μM). Control experiments were also carried out by exposing cells to medium alone or AS alone.

To investigate whether AS was increasing haem uptake due to a direct influence of nitroxyl on the ability of cells to incorporate haemin or because of a

direct chemical interaction of haemin and nitroxyl, experiments were carried out exposing cells to AS (250 μM) 5 min prior to addition of haemin. Considering that AS has a half-life of 2-3 min in physiological solutions, 5 min was estimated to be an adequate time before addition of haemin. Also, haem content was measured in renal epithelial cells, Girardi cells and RAW 264.7 macrophages exposed to haemin in the presence or absence of AS. The influence of hydroxyurea on endothelial haem uptake was investigated. Cells were incubated with haemin (15 μM) or three haemoglobins (HbA_0, HbS, methaemoglobin) in the presence or absence of hydroxyurea (300 μM) and haem content was measured after 2, 4 and 6 h.

4.3.2 Assay for determination of cellular haem content

The haem content was determined as described in section 2.10. Briefly, at the end of the incubation cells were washed twice with warm PBS, followed by addition of 1 ml formic acid to solubilise the endothelial layer. The haem concentration in the formic acid solution was measured spectrophotometrically at 398 nm ($\varepsilon = 1.56 \times 10^5\ M^{-1}cm^{-1}$). Haem uptake was expressed as pmol/well.

4.4 Results

4.4.1 Effect of AS on endothelial haem uptake elicited by haemoglobins

The experiments conducted here were performed with AS based on the assumption that, if any modulation of haem uptake was to be observed, we would be able to detect it within a short period of time because of AS's short half-life. Figure 4-1 shows that endothelial cells treated with AS did not exhibit changes in haem levels, while incubation with the different haemoglobins resulted in enhanced haem uptake. Interestingly, the pattern of haem uptake was dependent on the haemoglobin used. Specifically, exposure of cells to methaemoglobin showed a continuous incorporation of haem over the 6 h period (Figure 4-1 A), whereas HbA_0 caused a peak of haem uptake at 2 h followed by a fall over the next 4 h (Figure 4-1 B). In contrast, HbS did not change haem levels until 4 h of incubation and this was followed by a fall at 6 h (Figure 4-1 C). The data indicate that methaemoglobin elicited the highest increase in cellular levels, followed by HbS and HbA_0.

The effect of co-incubation of AS with haemoglobins on endothelial haem uptake depended again on the type of haemoglobin employed. As shown in Figure 4-1 A, haem incorporation at 2 and 4 h was similar between cells incubated with methaemoglobin alone or in combination with AS; however, after 6 h haem uptake was significantly lower with methaemoglobin plus AS than with methaemoglobin alone. In contrast, AS enhanced cellular haem levels in the presence of HbA_0 or HbS (Figure 4-1 B, C) at all time points considered. In particular, co-incubation of HbA_0 with AS showed enhanced haem uptake at 4 h

followed by a fall at 6 h. In contrast, HbS caused a continuous accumulation of haem in the presence of AS over the 6 h period.

Overall, these results indicate that the presence of AS enhanced haem uptake when cells were co-incubated with HbA_0 or HbS, but this effect was not reproduced with methaemoglobin.

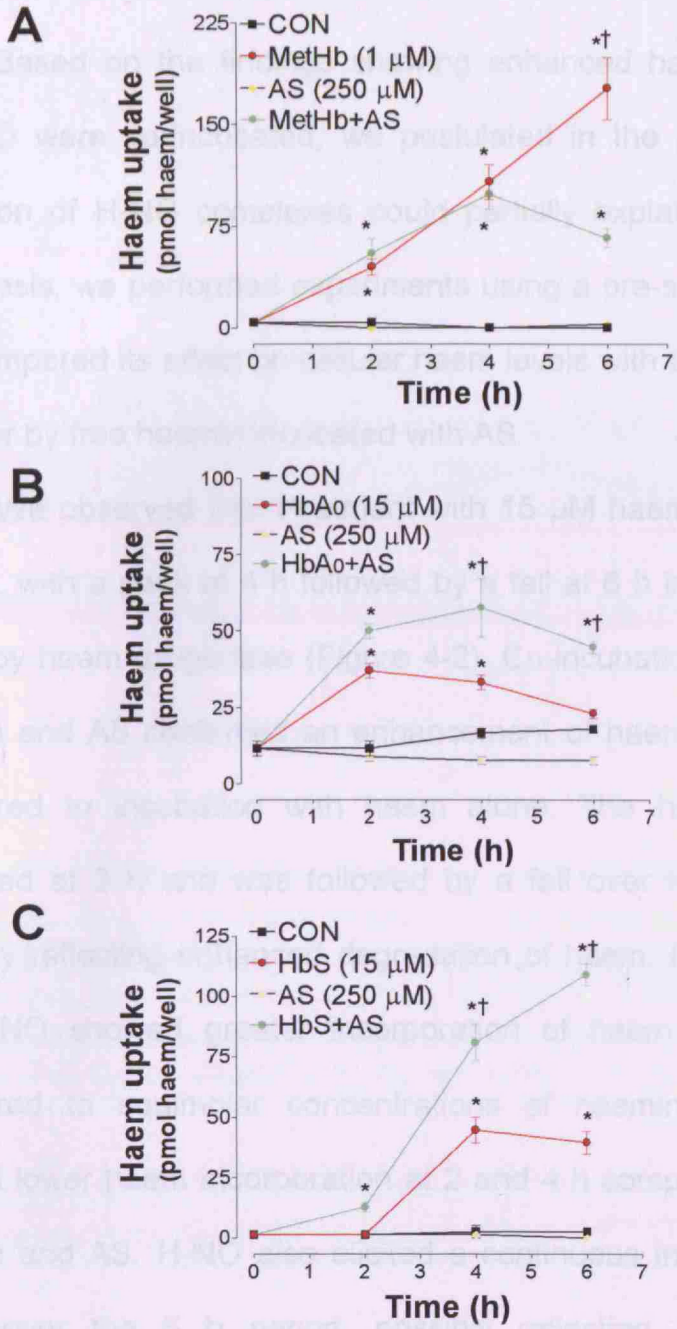


Figure 4-1. A nitroxyl generator synergizes with different haemoglobins to up-regulate endothelial haem uptake

For the haem uptake experiments, endothelial cells were exposed to medium alone (CON), Angeli's salt (AS) or MetHb in the presence or absence of AS and haem levels were assessed after 0, 2, 4 or 6 h incubation (A). Similar experiments were conducted with HbA_0 (B) and HbS (C). Data represent the mean±SEM of 6 independent experiments per group. * $P<0.05$ vs. CON; $^{\dagger}P<0.05$ vs. each different haemoglobin alone.

4.4.2 Comparison between haem-nitrosyl and haemin on changes of cellular haem levels

Based on the findings showing enhanced haem uptake when haemin and NO were co-incubated, we postulated in the previous chapter that the formation of H-NO complexes could partially explain this effect. To test this hypothesis, we performed experiments using a pre-synthesized H-NO complex and compared its effect on cellular haem levels with that elicited by free haemin alone or by free haemin incubated with AS.

We observed that treatment with 15 μM haemin alone enhanced haem uptake, with a peak at 4 h followed by a fall at 6 h indicative of degradation of haem by haem oxygenase (Figure 4-2). Co-incubation of endothelial cells with haemin and AS confirmed an enhancement of haem uptake at all time points compared to incubation with haem alone. The highest haem uptake was observed at 2 h and was followed by a fall over the remaining time points, possibly reflecting enhanced degradation of haem. Incubation of cells with 15 μM H-NO showed greater incorporation of haem at all time points when compared to equimolar concentrations of haemin alone. However, H-NO caused lower haem incorporation at 2 and 4 h compared to co-incubation with haemin and AS. H-NO also elicited a continuous increase in incorporation of haem over the 6 h period, possibly reflecting an impediment to haem degradation.

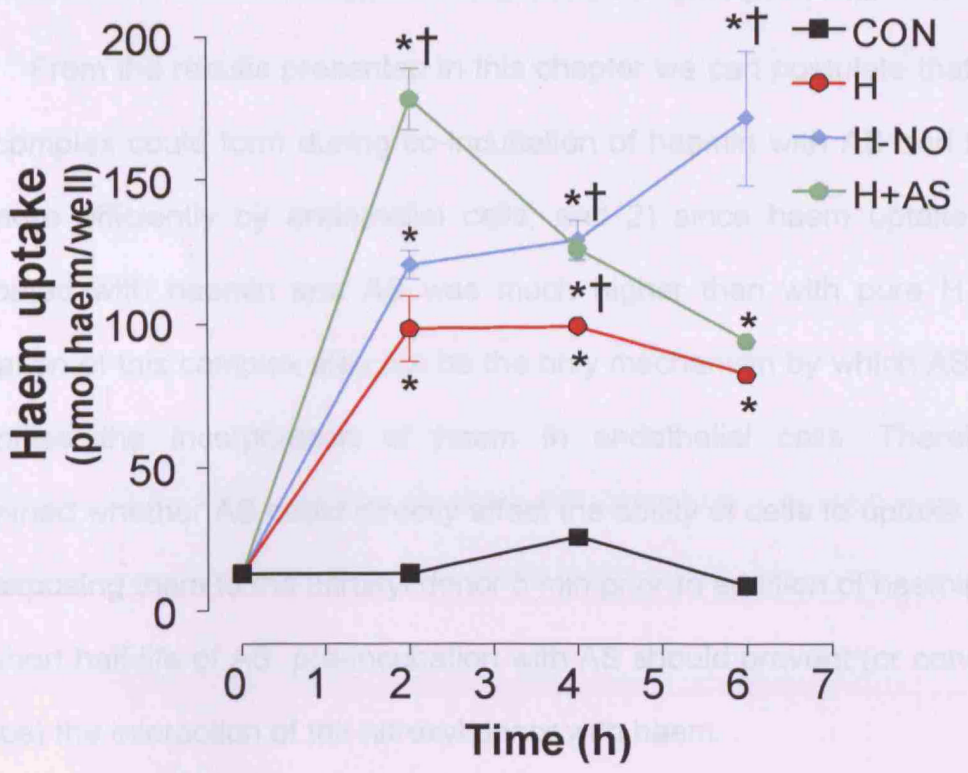


Figure 4-2. Effect of haem-nitrosyl complex (H-NO) on haem uptake

Cells were exposed to medium alone (CON), haemin (H, 15 μM), haemin in the presence of 250 μM AS (H+AS) or a H-NO complex (H-NO, 15 μM) and haem uptake was determined at 0, 2, 4 and 6 h as described in Materials and Methods. Data represent the mean±SEM of 5-6 independent experiments per group. * $P<0.05$ vs. CON; † $P<0.05$ vs. haemin alone.

4.4.3 Effect of bovine albumin on increased haem uptake mediated by AS

From the results presented in this chapter we can postulate that: 1) a H-NO complex could form during co-incubation of haemin with AS and be taken up more efficiently by endothelial cells; and 2) since haem uptake in cells incubated with haemin and AS was much higher than with pure H-NO, the formation of this complex may not be the only mechanism by which AS strongly magnifies the incorporation of haem in endothelial cells. Therefore, we examined whether AS could directly affect the ability of cells to uptake haem by pre-exposing them to the nitroxyl donor 5 min prior to addition of haemin. Due to the short half-life of AS, pre-incubation with AS should prevent (or considerably reduce) the interaction of the nitroxyl donor with haem.

Notably, we observed that the haem content of cells pre-incubated with AS followed 5 min later by addition of haemin was even higher than that measured when cells were incubated with haemin alone or haem plus AS (Figure 4-3 A). This observation strongly suggests that nitroxyl could possibly interact with cells or cell membranes and favour an increased transport of haem in the endothelial cell. Again, a peak of haem uptake was seen at 4 h followed by a fall at 6 h, reflecting the degradation of haem by haem oxygenase.

The effect of bovine albumin on haem uptake was also investigated. Albumin is a serum protein that binds haem non-specifically. Therefore, it was not surprising to observe that albumin decreased cellular haem incorporation in a concentration-dependent manner (Figure 4-3 B), as demonstrated previously in rat aortic tissue (Bui et al., 2004). AS was able to enhance haem uptake even in the presence of albumin. In fact, the pmol of haem/well measured at 2 h were 665.6 ± 28, 146.4 ± 5 and 263.9 ± 19 for haemin alone, haemin incubated with

5 μM albumin or haemin incubated with 5 μM albumin in the presence of AS, respectively. Unlike the previous experiments, the data presented in Figure 4-3 were obtained from cells incubated in serum-free medium in order to evaluate the real effect of AS or albumin on haem uptake without the interference of serum components.

In summary, the uptake of haem in endothelial cells can be influenced by pre-incubating cells with AS, with NO having a possible action on cellular membranes to enhance the incorporation of haem. The presence of albumin in the medium also reduces the level of haem incorporated into the cells.

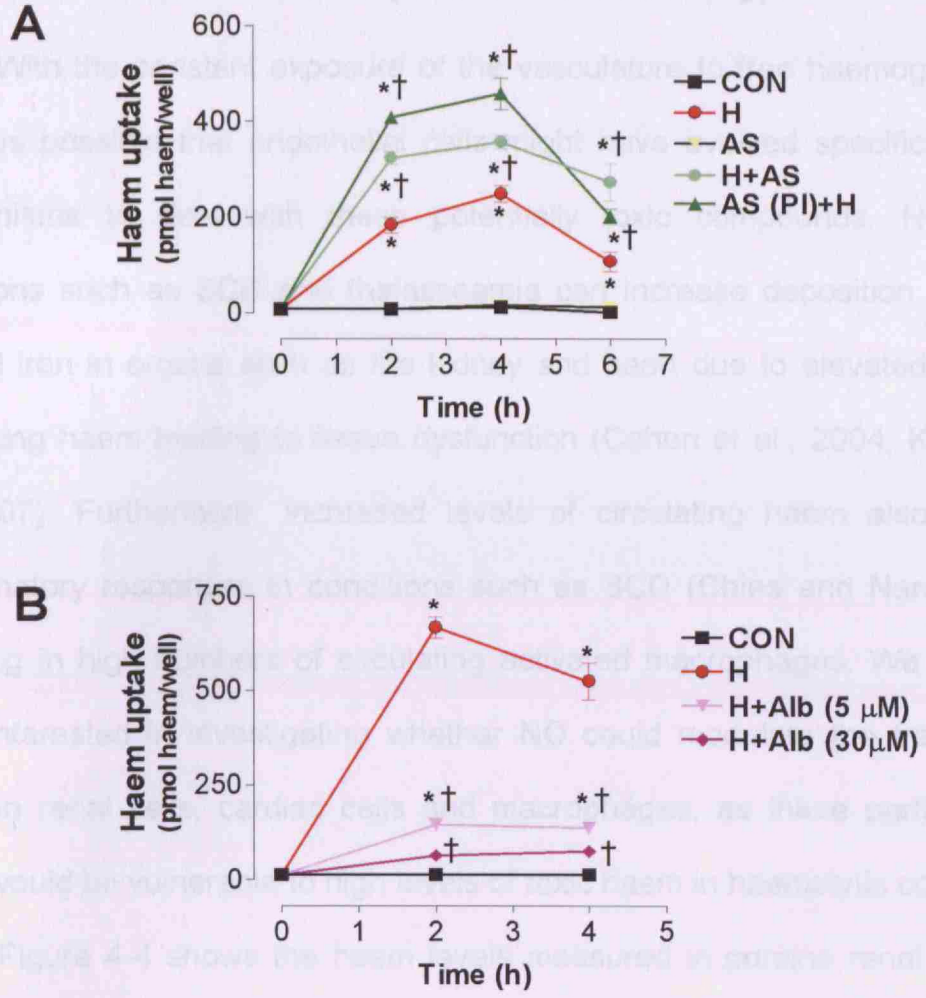


Figure 4-3. Pre-incubation with AS and bovine serum albumin modulate haem uptake by endothelial cells

(A) Cells were exposed to medium alone (CON), 15 μM haemin (H), 250 μM AS, haemin and AS (H+AS) or pre-incubated with AS for 5 min prior to addition of haemin (AS(PI)+H). Haem uptake was assessed at 0, 2, 4 and 6 h as described in Materials and Methods. (B) Cells were incubated in medium alone (CON), haemin (H, 15 μM) or haemin in the presence of 5 or 30 μM bovine albumin (H+Alb). Haem uptake was determined at 0, 2, 4 and 6 h. All sets of experiments were performed in the absence of foetal bovine serum to avoid interference with serum components. Data represent the mean±SEM of 5-6 independent experiments per group. * $P<0.05$ vs. CON; † $P<0.05$ vs. haemin alone.

4.4.4 Measurements of haem uptake in different cell types

With the constant exposure of the vasculature to free haemoglobin and NO, it is possible that endothelial cells might have evolved specific adaptive mechanisms to deal with these potentially toxic compounds. Haemolytic conditions such as SCD and thalasseamia can increase deposition of haem-derived iron in organs such as the kidney and heart due to elevated levels of circulating haem leading to tissue dysfunction (Cohen et al., 2004; Kirkizlar et al., 2007). Furthermore, increased levels of circulating haem also provoke inflammatory responses in conditions such as SCD (Chies and Nardi, 2001), resulting in high numbers of circulating activated macrophages. We therefore were interested in investigating whether NO could modulate the transport of haem in renal cells, cardiac cells and macrophages, as these particular cell types would be vulnerable to high levels of toxic haem in haemolytic conditions.

Figure 4-4 shows the haem levels measured in porcine renal epithelial cells (Figure 4-4 A), human adult atrial myoblast cells (Girardi cells) (Figure 4-4 B) and murine RAW 264.7 macrophages (Figure 4-4 C) following incubation with haemin in the presence or absence of AS. Treatment of cells with AS alone did not change the haem content of cells. In contrast, incubation with haemin alone enhanced haem uptake in the three cell types to varying degrees. Girardi cells showed the highest levels of haem incorporation. It is important to note that Girardi cells displayed an intrinsically higher haem content at basal conditions compared to renal cells and macrophages. This is explained by the fact that cardiac cells contain high levels of myoglobin and many mitochondria enclosing haem-dependent cytochromes. Co-incubation of the three cell types with AS and haemin increased the uptake of haem with the effect being more

pronounced in Girardi cells and macrophages. In fact, exposure of Girardi cells and macrophages to AS and haem doubled cellular haem incorporation when compared to haem alone, a result that was comparable to earlier experiments with endothelial cells.

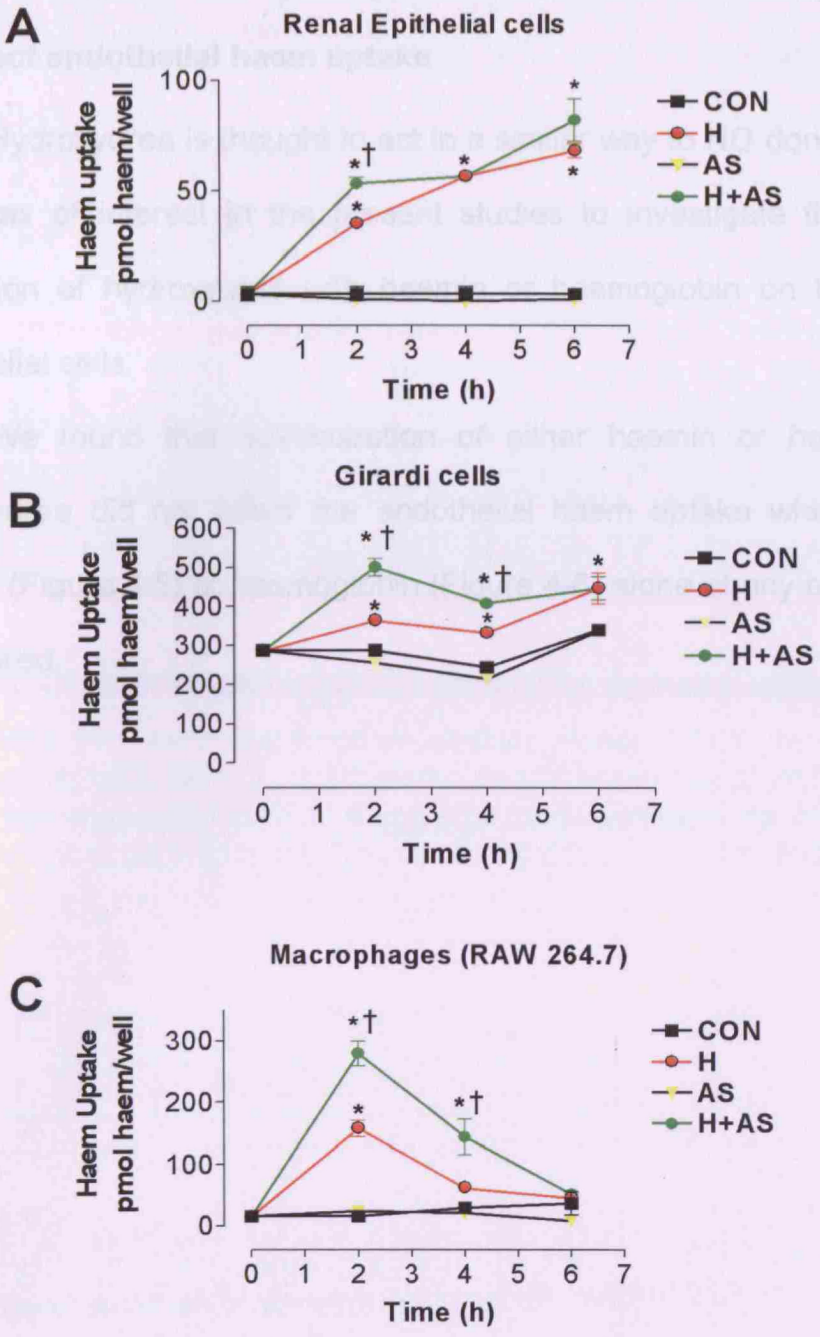


Figure 4-4. Haem uptake in different cell types

Renal epithelial cells (A), Girardi cells (B) and RAW 264.7 macrophages (C) were exposed to medium alone (CON), 15 μM haemin (H), AS, (250 μM) or haemin in the presence of AS (H+AS). Haem uptake was measured at 0, 2, 4 and 6 h. Data represent the mean±SEM of 5-6 independent experiments per group. * $P<0.05$ vs. CON; † $P<0.05$ vs. haemin alone.

4.4.5 Co-incubation of hydroxyurea with haemin or haemoglobin does not affect endothelial haem uptake

Hydroxyurea is thought to act in a similar way to NO donors (King, 2004) so it was of interest in the present studies to investigate the effect of co-incubation of hydroxyurea with haemin or haemoglobin on haem uptake in endothelial cells.

We found that co-incubation of either haemin or haemoglobin with hydroxyurea did not affect the endothelial haem uptake when compared to haemin (Figure 4-5) or haemoglobin (Figure 4-6) alone at any of the time points considered.

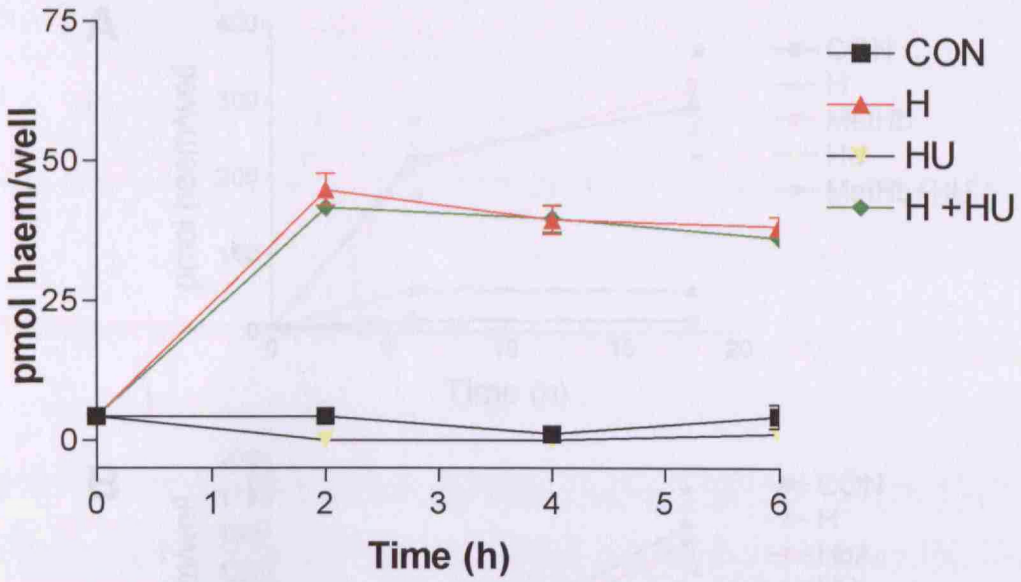


Figure 4-5. Effect of hydroxyurea and haemin on haem uptake

Endothelial cells were exposed to medium alone (CON), 15 μM haemin (H), hydroxyurea (HU, 300 μM) or haemin in the presence of HU (H+HU). Haem uptake was measured at 0, 2, 4 and 6 h. Data represent the mean±SEM of 5-6 independent experiments per group. * $P<0.05$ vs. CON; † $P<0.05$ vs. haemin alone.

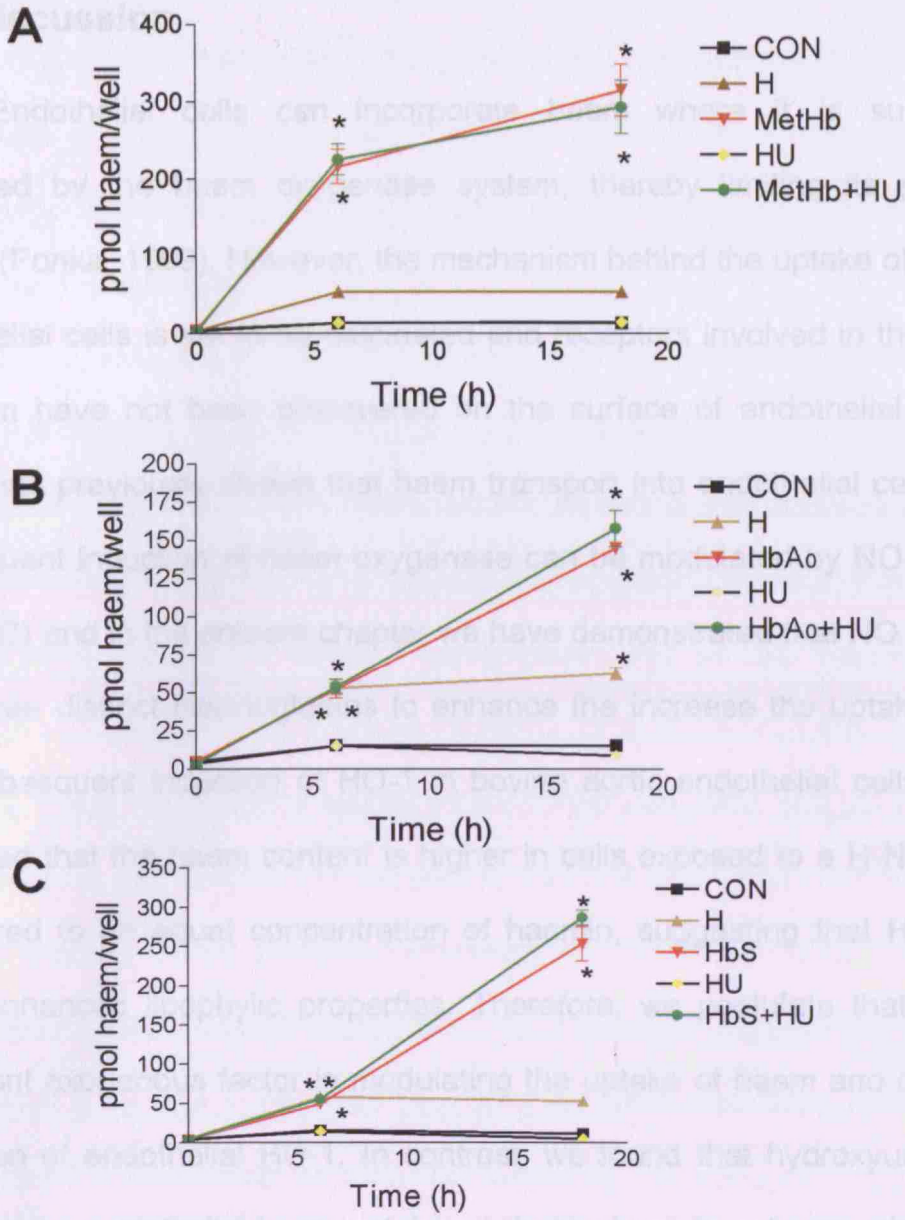


Figure 4-6. Effect of hydroxyurea and haemoglobins on haem uptake

Cells were exposed to medium alone (CON), 300 μM hydroxyurea (HU) or 1 μM methaemoglobin (MetHb) in the presence or absence of HU and haem levels were assessed after 0, 2, 4 or 6 h incubation (A). Similar experiments were conducted to measure haem uptake with 15 μM HbA_0 (B) and 15 μM HbS (C). Data represent the mean±SEM of 6 independent experiments per group. *P<0.05 vs. CON.

4.5 Discussion

Endothelial cells can incorporate haem where it is subsequently degraded by the haem oxygenase system, thereby limiting its pro-oxidant effects (Ponka, 1999). However, the mechanism behind the uptake of haem into endothelial cells is yet to be elucidated and receptors involved in the transport of haem have not been discovered on the surface of endothelial cells. Our group has previously shown that haem transport into endothelial cells and the consequent induction of haem oxygenase can be modulated by NO (Foresti et al., 2003) and in the present chapter we have demonstrated that NO synergizes with three distinct haemoglobins to enhance the increase the uptake of haem and subsequent induction of HO-1 in bovine aortic endothelial cells. We also observed that the haem content is higher in cells exposed to a H-NO complex compared to an equal concentration of haemin, suggesting that H-NO might have enhanced lipophylic properties. Therefore, we postulate that NO is an important exogenous factor in modulating the uptake of haem and consequent induction of endothelial HO-1. In contrast, we found that hydroxyurea did not enhance the endothelial haem uptake elicited by haemin or haemoglobins.

Although, all three haemoglobins synergized with NO to enhance endothelial haem uptake, methaemoglobin alone caused a continuous accumulation of cellular haem during the time course of our experiments. An explanation for this observation could be enhanced haem generation from methaemoglobin in comparison to the HbA_0 and HbS over the time period of the experiment. Exposure of cells to methaemoglobin in the presence of NO resulted in a fall in haem content after 4 h, indicating endogenous degradation of haem by haem oxygenase and suggesting that NO donors stimulate haem

catabolism. Following co-incubation of cells with NO and HbA_0 or HbS we observed enhanced haem uptake compared with the haemoglobins alone, but the profiles of haem incorporated differed between the two haemoglobins. The fact that the combination of NO and HbS promoted a continued accumulation of haem over 6 h whereas haem levels started to decrease after 4 h of incubation with NO and HbA_0 suggest that the interaction of the two haemoglobins with NO is different. Indeed, there is indication in the literature of an abnormal reactivity of HbS with NO which influences vascular functions (Pawloski et al., 2005) . Since the interaction of NO and reduced haemoglobin results in the production of methaemoglobin and nitrate, we envision that in our experiments the oxidation of HbA_0 and HbS leads to formation of methaemoglobin, which easily releases haem that is more readily incorporated into cells. NO could also bind haemoglobins and form H-NO complexes that are easily taken up by cells. Our findings that the H-NO complex enhanced the uptake of haem in endothelial cells and induction of HO-1 compared to haemin alone support this hypothesis. It is conceivable that H-NO may bind to potential membrane receptors with a higher affinity compared to haemin, although we recognise this to be a speculative idea. Alternatively, H-NO and haemin might incorporate into cells in a similar manner but H-NO may be more difficult to degrade by HO-1 than haemin alone. This interpretation is supported by the results showing that H-NO only caused marginal enhancement in BR levels compared to haemin alone and has been proposed previously by another group (Juckett et al., 1998). Indeed, Juckett and co-workers have suggested that H-NO complexes aggregate in microsomal membranes to sequester free haem intracellularly, protecting it from haem oxygenase-mediated catabolism, which was reflected in their observation

that H-NO inhibits HO-1 activity. In contrast, we have seen that pure H-NO enhances HO-1 induction compared to haemin alone. Intriguingly, high levels of H-NO complexes have been found to form in ischaemic heart tissue, where nitrite-mediated H-NO formation occurs progressively during ischaemia (Tiravanti et al., 2004). In this context, haem molecules, through formation of H-NO, act as potential stores of NO, with subsequent activation of NO signalling pathways on reperfusion. It is possible that the formation of H-NO is a mechanism to limit the potential oxidative and nitrosative damage exerted by free haem and NO, especially in pathophysiological conditions characterized by raised levels of both these potential hazardous substances.

Our findings have shown that H-NO induces HO-1 and increases haem levels to a greater extent than haemin alone but to a lower degree than haemin plus AS. This suggests that H-NO formation is implicated in the HO-1 induction and haem uptake observed upon co-incubation of haemoglobins and NO, but also emphasises that such a mechanism cannot fully explain this phenomenon. Our results showing that pre-incubation of endothelial cells with AS increased the ability of cells to uptake haem, suggest that NO affects the surface of the endothelial cell membrane, either in modulating the binding properties of potential haem transport proteins (receptors or carrier proteins) or directly influencing the permeability of cellular membrane thus stimulating the incorporation of haem into the cell.

Of interest, we found that the carrier protein albumin reduced the uptake of haem by endothelial cells and that the addition of AS still enhanced haem uptake in the presence of albumin. This suggests the mechanisms underlying

AS-mediated increase in cellular haem incorporation occur even in a more complex biological solution containing albumin.

Significantly, we found that AS could also enhance the incorporation of haem in renal epithelial cells, Girardi cells and macrophages in a comparable manner to that observed in endothelial cells. These data suggest that the effect of NO on enhancing cellular haem uptake is not limited to endothelial cells but could represent an adaptive mechanism for all tissue exposed to high levels of haem. Indeed, in haemolytic diseases such as SCD, tissue and organ damage results from high levels of free toxic haem (Nath et al., 2001a; Redding-Lallinger and Knoll, 2006) and our observations of NO-enhanced cellular transport in renal and cardiac cells may provide a strategy for protection against haem-mediated injury and organ dysfunction in haemolytic diseases. Elevated levels of haem also elicit inflammatory responses in conditions such as SCD (Chies and Nardi, 2001), and the enhanced incorporation of haem by NO we observed in macrophages could provide an additional mechanism to modulate inflammatory responses in haemolytic states through the increased removal of the pro-inflammatory haem molecule.

Our initial hypothesis that hydroxyurea could influence haem uptake by virtue of its NO-donor properties was not confirmed in the present study, nor with haemin or with the different haemoglobins analysed. These results were partly expected, since co-incubation of hydroxyurea with haemoglobin did not produce an evident increase (with the exception of a slight effect with HbS) in NO release from hydroxyurea as measured with the NO electrode (see previous chapter). We can also postulate from this set of experiments that the synergism in haem oxygenase activation observed with simultaneous exposure of haemin

or haemoglobin with hydroxyurea (see previous chapter) is independent from effects on haem. Clearly, there are a series of unidentified mechanisms explaining the interaction of hydroxyurea and haemoglobins that need to be explored.

In conclusion, the interaction of haemin/haemoglobin and NO increases the uptake of haem in endothelial cells as well as the activity of haem oxygenase. This effect has also been seen in a variety of cell types, suggesting a novel role for NO in the modulation of haem transport. The synergism elicited by NO and haemoglobin/haemin on the induction of HO-1 may have a potential role in the treatment of conditions characterized by increased levels of haem and NO such as SCD, haemolytic or haemorrhagic shock.

Chapter 5. Effect of sickle blood on endothelial haem oxygenase

5.1 Introduction

SCD arises from a single point mutation in the ß-globin gene (glutamic acid is replaced by valine), which subsequently results in the production of HbS. Deoxygenation causes HbS to polymerise and red blood cells to take on the characteristic sickle shape. This leads to erythrocyte rigidity, membrane damage, haemolysis and resulting damage to tissues and organs (Bunn, 1997). The most successful strategy to prevent red blood cell sickling is by promoting increased HbF production in SCD patients, thus reducing the intracellular polymerization of HbS and the sickling effect. This is the mechanism by which hydroxyurea has been selected as the current preferential pharmacological approach to ameliorate SCD. However, even under hydroxyurea treatment, sickle patients still exhibit a persistent inflammatory state as well as haemolysis (Jison et al., 2004), indicating the need to find new alternative therapies for the disease. HO-1 induction has been demonstrated in various cell types in SCD, such as leukocytes (Jison et al., 2004), kidney tissue and circulating endothelial cells (Nath et al., 2001a) and limited evidence in the literature suggests that the biproducts of haem degradation by haem oxygenase exert important actions in SCD. In fact, Beutler demonstrated in 1975 that CO significantly prolongs red cell survival in SCD (Beutler, 1975) and BR was more recently reported to be a significant anti-oxidant in the plasma of SCD patients (Dailly et al., 1998). Taken together, these preliminary observations suggest that investigations into the role

of HO-1 in SCD may have important implications for understanding the pathology of SCD and for future therapeutic strategies.

We aimed in the present chapter to examine the effect of blood from sickle patients on haem oxygenase induction in endothelial cells and to identify factors, such as treatment regimen or patient characteristics that influence this effect.

5.2 Objectives

- To investigate whether whole sickle blood affects haem oxygenase activity level in endothelial cells

- To assess the effect of the single blood components, sickle plasma and red blood cells, on haem oxygenase activity in endothelial cells

- To examine whether hypoxia influences the increase in endothelial haem oxygenase activity elicited by sickle blood or haemoglobin

- To study the effect of hydroxyurea on sickle blood or haem-mediated increase in haem oxygenase under normoxic and hypoxic conditions

- To identify whether treatment regimen, number and timing of vaso-occlusive crises as well as patient characteristics (gender, age, haemoglobin, BR and LDH levels) are correlated with haem oxygenase activity

5.3 Experimental protocol

The detailed methods used in the experiments described in the following section can be found in the Materials and Methods chapter. All stocks were freshly prepared. Hydroxyurea stock solutions (10 mM) were prepared in distilled water. A stock solution of DETA-NO (10 mM) was prepared in 0.01 M NaOH. Whole blood was collected fresh in EDTA tubes from sickle patients and controls as described below.

5.3.1 Patient population

One hundred and ten patients with SCD were recruited for the study. Patients attended the Sickle Cell Clinic at Central Middlesex Hospital (London, UK) and were asked to provide a blood sample for research purposes. Eligibility criteria included all forms of SCD (genetic backgrounds: HbSS, HbSC, and HbSβThal), patients over the age of 12 months, and patients either receiving or not receiving hydroxyurea treatment. Patient characteristics can be found in the results section (Table 5-1). Information provided on the samples included: SCD genotype, whether the patient was receiving hydroxyurea or transfusion therapy, the current disease status (steady state or active crisis), the date of the last transfusion or last crisis, the number of crises in the last year, and LDH, BR and haemoglobin levels. LDH and BR levels were measured by the haematology laboratory at Central Middlesex Hospital using an Abbott Architect ci8200 integrated analyzing system and haemoglobin levels were measured with a Beckman Coulter LH 750 analyser using standardised assay kits. The study was approved by the Central Office of Research Ethics Committees (COREC). Plasma from a subset of 13 subjects from the patient population was tested for albumin, urea, creatinine, cholesterol, high density lipoprotein

cholesterol (HDL-C), total BR and uric acid with standardised kits using a ILab 650 Clinical Chemistry system at Northwick Park Institute of Medical Research. Twenty healthy individuals not suffering from SCD were recruited as a control population for comparison (Table 5-2).

5.3.2 Incubation of cells with reagents

Sickle blood was collected in EDTA-coated tubes and stored at 4°C until required; blood was used within 24 h from collection. In some experiments sickle or normal blood components were used, therefore whole blood was separated by centrifugation at 100 *g* for 5 min. The plasma was then removed and red blood cells washed with PBS before use. Red blood cells were used immediately, whereas plasma was stored at -80°C until required.

Initially, the ability of sickle blood to affect endothelial haem oxygenase activity at 21% oxygen was investigated. Preliminary experiments were designed to explore the percentage of blood in medium which would elicit endothelial haem oxygenase induction. Endothelial cells were incubated with 5, 10 and 20% whole normal blood in media for 6 h and 20% whole blood was found to be sufficient to induce haem oxygenase activity. Therefore, all subsequent experiments were conducted using medium containing 20% whole sickle or control blood for 6 or 18 h. In the next set of experiments, we explored the effect of blood components, plasma or red blood cells, on endothelial haem oxygenase activity. Endothelial cells were exposed for 6 or 18 h to either 10% plasma or washed red blood cells (from sickle or control blood) in medium.

A primary feature characterizing SCD is polymerization of deoxygenated HbS which leads to deformation of erythrocytes and haemolysis (Bunn, 1997). Therefore, the influence of hypoxia on the ability of sickle blood to induce haem

oxygenase activity was examined by exposing endothelial cells to 20% whole sickle blood for 18 h under low oxygen tension (1%O_2). These experiments were also carried out in the presence or absence of hydroxyurea (300 μM).

Experiments were also conducted by incubating cells with 15 μM HbA_0 or HbS at 1% O_2 for 18 h in the presence or absence of hydroxyurea (300 μM). It has recently been shown that NO synergises with different haemoglobins to stimulate endothelial HO-1 expression (Foresti et al., 2003). In the context of the present study, it was assessed whether a similar phenomenon could be replicated with sickle blood. Experiments were performed by incubating cells with 20% whole blood in the presence or absence of 250 μM S-nitrosoglutathione (GSNO, 6 h) or 300 μM hydroxyurea (18 h). Some of the experiments described above were also performed for determination of HO-1 protein levels.

5.3.3 Haem oxygenase activity assay

Haem oxygenase activity was determined in endothelial cells at different times after treatment as described in section 2.7. Briefly, harvested cells were subjected to three cycles of freeze-thawing before addition to a reaction mixture containing NADPH, rat liver cytosol as a source of BVR, and the substrate haemin. The reaction was conducted at 37 °C in the dark for 1 h, terminated by the addition of 1 ml chloroform, and the extracted BR was calculated by the difference in absorbance between 464 and 530 nm (ε = 40 $mM^{-1}cm^{-1}$).

5.3.4 Western-blot technique for detection of HO-1 protein expression

Samples of endothelial cells also underwent Western immunoblot analysis, as described in section 2.9. Briefly, an equal amount of proteins (30

μg) for each sample was separated by SDS-polyacrylamide gel electrophoresis, transferred overnight to nitrocellulose membranes, and the non-specific binding of antibodies was blocked with 3% non-fat dried milk in PBS. Membranes were then probed with a polyclonal rabbit anti-HO-1 antibody (Stressgen, Victoria, Canada) (1:1,000 dilution in Tris-buffered saline, pH 7.4). After three washes with PBS containing 0.05% (v/v) Tween 20, blots were visualized using an amplified alkaline phosphatase kit from Sigma (Extra-3A) and subjected to densitometric analysis. To ensure equal loading, cell samples also underwent Western-blot analysis for the house-keeping gene β-actin.

5.3.5 Statistical analysis

One way ANOVA was performed to study the effects of sickle blood exposure on endothelial haem oxygenase activity. Two-sided probability values were calculated by unpaired t-test for comparison between patients on different treatment regimens or in disease states. Pearson's correlation was performed to evaluate the effect of patient characteristics (gender, age, haemoglobin, BR or LDH levels) on endothelial haem oxygenase activity after exposure to sickle blood. Measurements shown are mean±SEM. Analysis was performed with Prism 4 (Graphpad Software).

5.4 Results

The results will be subdivided into experimental and analytical sections. In the first section, data from experiments investigating the endothelial haem oxygenase response from exposure to sickle blood under different conditions will be presented. The second section will involve further analysis on the relationship between haem oxygenase activity and patient characteristics such as disease state, treatment regimen, gender, age and blood parameters (haemoglobin, BR and LDH levels).

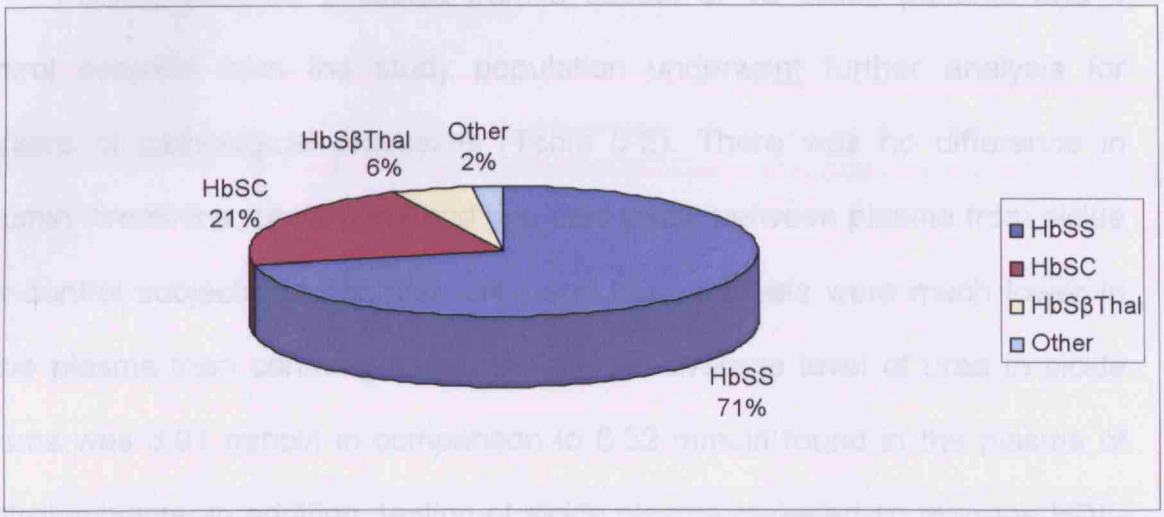
5.4.1 Experimental results

5.4.1.1 Patient characteristics

Within the sickle patient population, a variety of sickle variants were present (Figure 5-1), with the most prevalent trait being HbSS (71%), followed by HbSC (21%) and HbSβThal (6%). A small minority of patients had minor sickle traits, hereditary persistence of foetal haemoglobin (HPFH) and SD disease, and represented only 2% of the study population. The profile of sickle variants in the recruited sickle population reflects that reported in the wider sickle population in that approximately two thirds (66%) of sickle cases are characterised by the HbSS genotype (Redding-Lallinger and Knoll, 2006), followed in order of frequency by HbSC and HbSβThal. The average age within the sickle population and controls was also comparable (see Table 5-1) at 33±13 and 36.6±4.75, respectively. There was also a similarity in the gender profile between the two populations, with 65% of the sickle and control groups being female. However, 8% of the sickle group recruited for the study were children whereas all the controls subjects were adults.

As severity of SCD within the individual and within the wider sickle population can vary over time, there was an expected variation in the disease state and treatment regimens undertaken by the recruited sickle patients. At the time of blood collection for the present study, 22% of sickle patients were experiencing an acute crisis, 20% were undertaking hydroxyurea treatment and 5% were receiving transfusion therapy.

Figure 5-1. Breakdown of sickle variants in SCD patient population



After the collection of blood samples from both the control and sickle groups, blood was immediately tested for haemoglobin, LDH and BR levels. As shown in Table 5-1, haemoglobin levels were significantly lower in the sickle group (10.6 mg/dl) compared to control subjects (13.7 mg/dl). In addition, LDH and BR levels were significantly raised in the sickle population compared to the control group. The average BR level measured in sickle patients blood was 51.6 μM compared to 7.82 μM detected in control blood. Furthermore, average blood LDH levels in sickle patients were 398 U/l in comparison to 189 U/l found in controls.

Table 5-1. Patients characteristics

	Control	SCD	*P**
No. of patients	20	110	
Age, mean±SD (%)	36.6±4.56	33±13	
Adult/child ratio (%)	20 (100%) / 0 (0%)	101 (92%) / 9 (8%)	
Gender, female/male ratio (%)	13 (65%) / 7 (35%)	71 (65%) / 39 (35%)	
Hb,mg/dl, mean±SD	13.71±1.07	10.6±6.2	P<0.0001
BR,μM, mean±SD	7.82±2.7	51.6±46.5	P<0.0001
LDH, Units/l, mean±SD	189±71.21	398±187	0.0286
Patients on hydroxyurea		22 (20%)	
Patients on transfusion		5 (5%)	
Disease state, acute crisis/steady state		24 (22%) / 86 (78%)	

*P value is for the comparison of sickle patients versus control subjects.

Plasma samples collected from a subset of 13 sickle patients and 4 control subjects from the study population underwent further analysis for markers of pathological processes (Table 5-2). There was no difference in albumin, creatinine, cholesterol and uric acid levels between plasma from sickle and control subjects. In contrast, urea and HDL-C levels were much lower in sickle plasma than control plasma. In fact, the average level of urea in sickle plasma was 3.91 mmol/l in comparison to 6.32 mmol/l found in the plasma of control subjects. In addition, testing of sickle plasma revealed an average HDL-C of 0.97 mmol/l compared to 1.69 mmol/l measured in control plasma. Within our subset of sickle patients there were some patients who were undertaking hydroxyurea treatment. Therefore, the results obtained from testing sickle plasma for pathological markers were further analysed to examine possible differences that may exist between patients receiving or not receiving hydroxyurea treatment. However, from the limited number of patients examined it appears that hydroxyurea does not influence the levels of plasma pathological markers, as no difference was observed between samples collected from sickle patients receiving or not receiving treatment.

Table 5-2. Plasma analysis of sickle patient subset

	CON (n=4)	SCD (n=13)	SCD + HU (n=3)	SCD – HU (n=10)	*P**	*P†*	*P‡*	*P§*
Albumin,g/l, mean±SD	44.7±4.4	41.31±6.3	41.8±2.2	41.2±7.2	0.3309	0.3488	0.3780	0.8845
Urea,mmol/l, mean±SD	6.32±1.2	3.91±1.62	3.7±0.5	3.97±1.86	0.0161	0.0194	0.0398	0.8134
Creatinine, µmol/l mean±SD	87±14.1	85.3±21.7	79±18	87.1±23.3	0.8868	0.5526	0.9938	0.6093
Cholesterol, mol/l, mean±SD	5.53±0.6	4.52±1.15	4.2±0.9	4.6±1.25	0.1212	0.0657	0.1961	0.6117
HDL-C,mmol/l, mean±SD	1.69±0.4	0.97±0.33	0.94±0.23	0.98±0.36	0.0021	0.0295	0.0066	0.8741
Uric Acid,µmol/l, mean±SD	326±72.6	379±110.0	397±167	374±98	0.3785	0.4711	0.3954	0.7675

*P** value is for the comparison of sickle patients (SCD) versus control subjects (CON).
P† value is for the comparison of sickle patients receiving hydroxyurea (SCD + HU) versus control subjects (CON).
P‡ value is for the comparison of sickle patients not receiving hydroxyurea (SCD - HU) versus control subjects (CON).
P§ value is for the comparison of sickle patients receiving hydroxyurea (SCD + HU) versus patients not receiving hydroxyurea (SCD-HU).

5.4.1.2 Sickle blood induces haem oxygenase in endothelial cells

Initially, experiments were performed to establish the concentration of whole normal blood required to elicit any increase in endothelial haem oxygenase activity. As blood samples would be limited, we endeavoured to devise a protocol which would allow the use of small quantities of blood but still produce reproducible outcomes in terms of endothelial haem oxygenase activity. Endothelial cells were incubated for 6 h with 5, 10 and 20% whole blood solution prepared in culture medium and significant induction of endothelial haem oxygenase activity was observed with 20% whole normal blood compared to control (Figure 5-2). Therefore, we conducted all subsequent experiments incubating cells with 20% whole sickle or normal blood solutions.

As blood from sickle patients regularly undergoes haemolysis and liberates large amounts of haemoglobin into blood plasma (Reiter et al., 2002), we examined the possibility that sickle blood could induce endothelial haem oxygenase. Exposure of endothelial cells to 20% whole normal blood did not significantly affect haem oxygenase activity after 6 or 18 h incubation (Figure

5-3 A), even though our preliminary experiments with whole control blood showed a small increase in haem oxygenase activity following 6 h exposure. In contrast, 20% whole sickle blood, whether obtained from the HbSS or HbSC genotype, significantly increased endothelial haem oxygenase activity compared to control cells (incubated with medium alone) or cells treated with 20% whole normal blood. However, a difference in the magnitude of haem oxygenase induction elicited by sickle blood was observed between the two sickle genotypes. Specifically, exposure of endothelial cells to 20% HbSS sickle blood showed a more pronounced enhancement of endothelial haem oxygenase activity when compared to incubation with 20% HbSC sickle blood at both 6 and 18 h. However, incubation with sickle blood from either genotype caused a much greater induction of haem oxygenase activity at 18 h than at 6 h, possibly reflecting a rise in haemolysis over time with release of haem from haemoglobin.

In further experiments we investigated which components of blood (i.e. plasma or red blood cells) were responsible for eliciting the enhanced haem oxygenase response in endothelial cells. We observed no differences in haem oxygenase activity following incubation with 10% normal or sickle plasma for 6 h and 18 h (Figure 5-3 B), suggesting that there were no constituents in the plasma of sickle patients that can directly promote an increase in endothelial haem oxygenase activity. However, exposure of endothelial cells to separated red blood cells from sickle or normal blood for 6 and 18 h, showed an increase in haem oxygenase activity compared with control (Figure 5-3 C). Furthermore, sickle red blood cells induced endothelial haem oxygenase activity to a greater extent than normal red blood cells. It should be noted that haem oxygenase

activity levels were slightly higher after incubation with normal red blood cells than with normal whole blood. Conversely, the extent of haem oxygenase induction was more pronounced with whole sickle blood than sickle red blood cells.

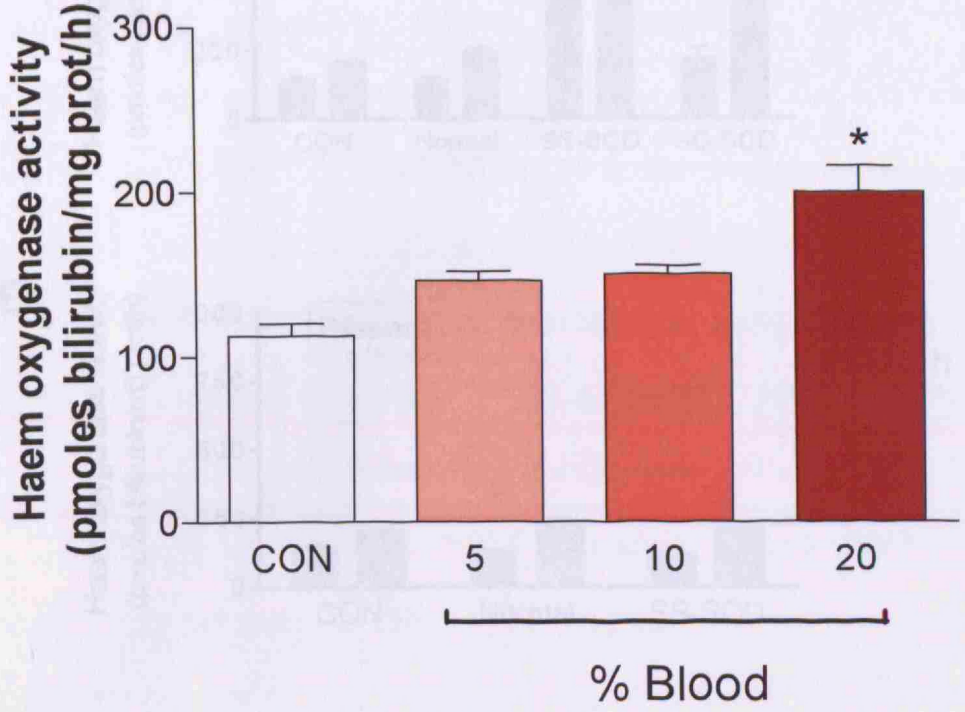


Figure 5-2. Endothelial haem oxygenase activity elicited by whole blood solutions

Endothelial cells were exposed to 5, 10 and 20% whole normal blood solution prepared in culture medium for 6 h. Cells were also exposed to medium alone (CON). Haem oxygenase activity measurements were performed as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. CON.

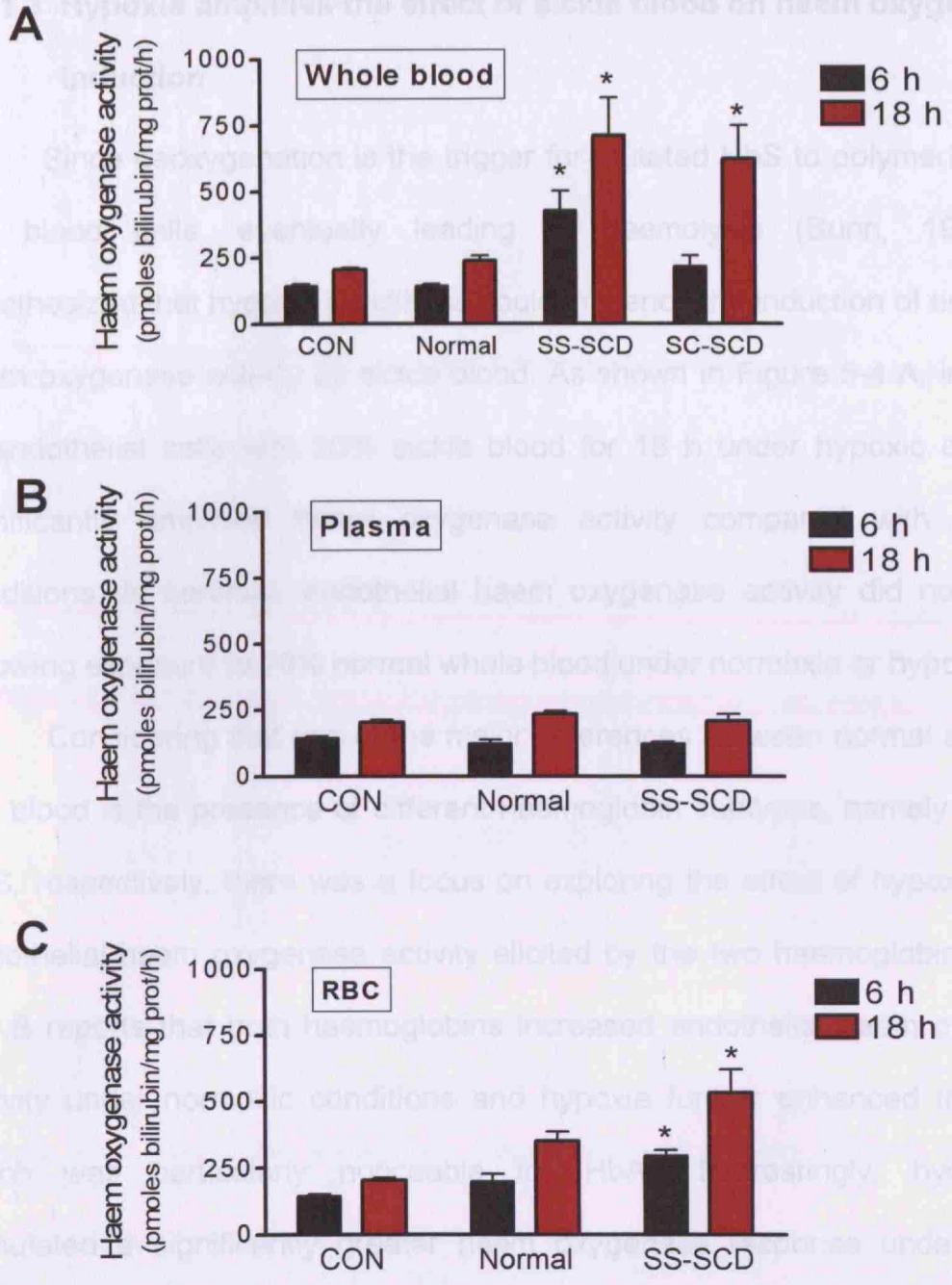


Figure 5-3. Sickle blood induces haem oxygenase in endothelial cells

Endothelial cells were exposed for 6 and 18 h to: (A) medium containing 20% whole sickle HbSS (SS-SCD) or HbSC (SC-SCD)) or control blood (Normal); (B) medium containing 10% plasma separated from sickle or control blood and (C) medium containing 10% washed red blood cells (RBC) separated from whole sickle or control blood. Cells were also incubated in medium alone (CON). Haem oxygenase activity was measured as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. CON.

5.4.1.3 Hypoxia amplifies the effect of sickle blood on haem oxygenase induction

Since deoxygenation is the trigger for mutated HbS to polymerise inside red blood cells, eventually leading to haemolysis (Bunn, 1997), we hypothesized that hypoxic conditions could influence the induction of endothelial haem oxygenase activity by sickle blood. As shown in Figure 5-4 A, incubation of endothelial cells with 20% sickle blood for 18 h under hypoxic conditions significantly amplified haem oxygenase activity compared with normoxic conditions. In contrast, endothelial haem oxygenase activity did not change following exposure to 20% normal whole blood under normoxia or hypoxia.

Considering that one of the major differences between normal and sickle cell blood is the presence of different haemoglobin subtypes, namely HbA and HbS, respectively, there was a focus on exploring the effect of hypoxia on the endothelial haem oxygenase activity elicited by the two haemoglobins. Figure 5-4 B reports that both haemoglobins increased endothelial haem oxygenase activity under normoxic conditions and hypoxia further enhanced this effect, which was particularly noticeable for HbA_0. Interestingly, hydroxyurea stimulated a significantly greater haem oxygenase response under hypoxic conditions than under normoxia. The drug also intensified the increased haem oxygenase activity and HO-1 expression (Figure 5-5) exerted by HbA_0 and HbS under hypoxic conditions. Therefore, it appears that under hypoxic conditions, hydroxyurea synergises with free haemoglobin to induce endothelial haem oxygenase activity.

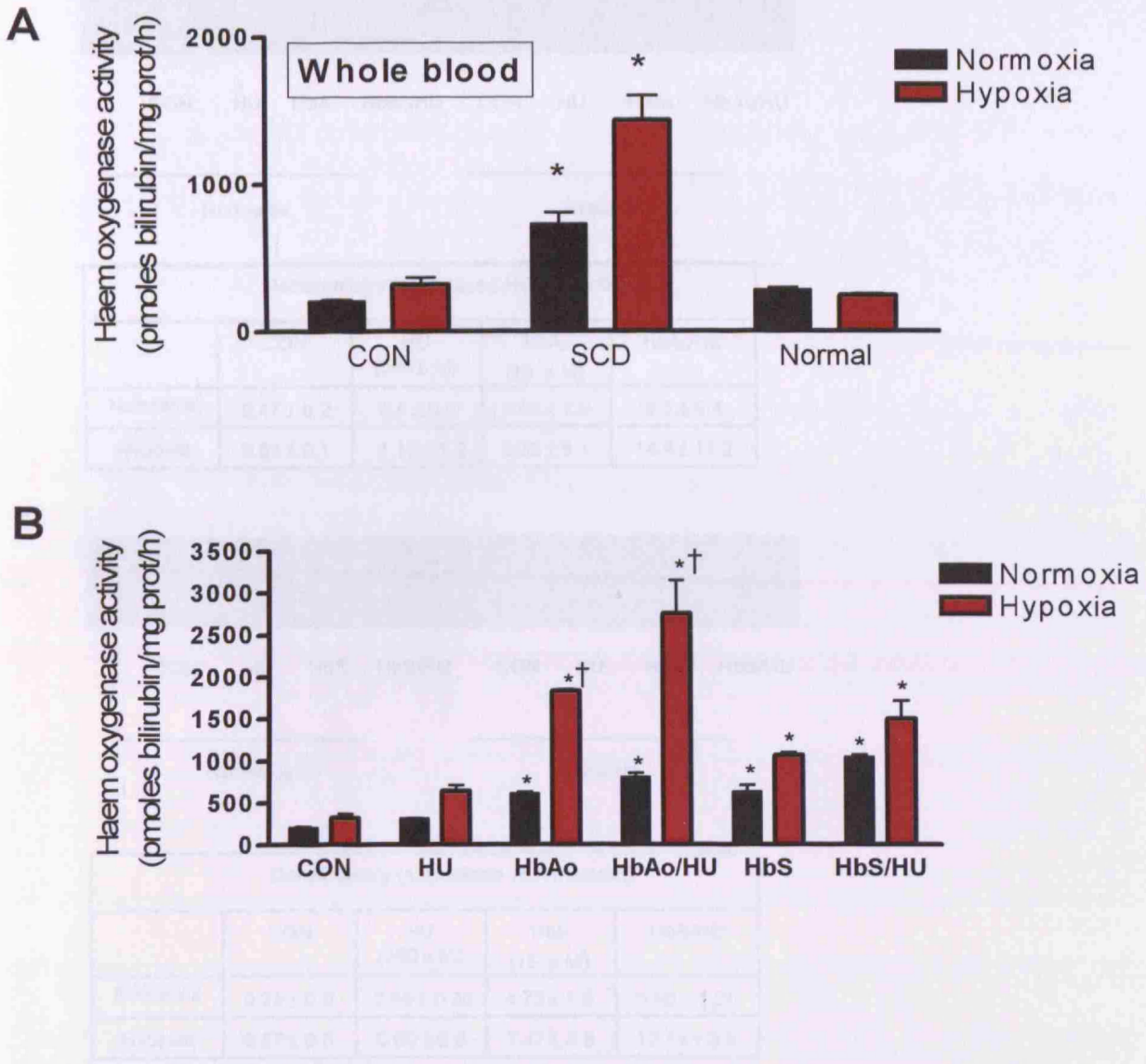


Figure 5-4. Hypoxia amplifies the effect of sickle blood on haem oxygenase induction in endothelial cells

Endothelial cells were exposed for 18 h to (A) medium containing 20% whole sickle blood (SCD) or control blood (Normal) at 1% O_2 (Hypoxia) or 21% O_2 (Normoxia) and (B) 15 μM HbA_0 or HbS at 1 or 21% O_2 in the presence or absence of 300 μM hydroxyurea (HU). Cells were also incubated in medium alone (CON). Haem oxygenase activity was measured as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. CON or † $P<0.05$ vs. Normoxia.

A

CON HU HbA_0 HbA_0/HU CON HU HbA_0 HbA_0/HU

Normoxia Hypoxia

Densitometry (normalised HO-1/β-actin)				
	CON	HU (300 μ M)	HbA_0 (15 μ M)	HbA_0/HU
Normoxia	0.47±0.2	0.8±0.6	3.86±2.8	9.0±5.4
Hypoxia	0.63±0.1	1.12±1.2	8.38±5.1	14.4±11.2

B

CON HU HbS HbS/HU CON HU HbS HbS/HU

Normoxia Hypoxia

Densitometry (normalised HO-1/β-actin)				
	CON	HU (300 μ M)	HbS (15 μ M)	HbS/HU
Normoxia	0.29±0.0	0.68±0.38	4.73±1.5	5.60±1.21
Hypoxia	0.67±0.0	0.69±0.0	7.47±2.8	12.14±3.5

Figure 5-5. Hypoxia amplifies the effect co-incubation of hydroxyurea with haemoglobin on HO-1 expression in endothelial cells

Endothelial cells were exposed for 18 h to (A) 15 μM HbA_0 at 1 or 21% O_2 in the presence or absence of 300 μM hydroxyurea (HU) and (B) 15 μM HbS at 1 or 21% O_2 in the presence or absence of HU (300 μM). Cells were also incubated in medium alone (CON). The expression of HO-1 protein was determined by Western blot technique as described in Materials and Methods. Each image is representative of three independent experiments and the tables report the mean arbitrary units of the results obtained by densitometric analysis of the bands (densitometry normalised to β-actin).

5.4.1.4 Co-incubation of NO donors with sickle blood does not affect induction of haem oxygenase

We explored whether co-incubation of endothelial cells with 20% whole blood and NO donors would affect haem oxygenase activity. As shown in Figure 5-6 A, 250 μM GSNO alone or 20% sickle whole blood induced haem oxygenase activity; however, GSNO in combination with sickle blood did not further enhance endothelial haem oxygenase enzymatic activity. A similar outcome was also obtained when hydroxyurea was co-incubated with sickle blood, despite the potential NO-releasing capacities of hydroxyurea (Figure 5-6 B). It was observed that incubation with 300 μM hydroxyurea alone produced a small increase in endothelial in haem oxygenase activity (Figure 5-6 B); however, co-incubation with sickle blood did not enhance the endothelial haem oxygenase activity elicited by sickle blood alone. As we have observed a hydroxyurea-mediated amplification of haem oxygenase activity under hypoxic conditions, it could be postulated that under hypoxia hydroxyurea may act differently when co-incubated with blood. Although exposure of cells to sickle blood under hypoxic conditions greatly enhanced haem oxygenase activity compared to normoxia, addition of hydroxyurea did not further enhance the activity (Figure 5-7).

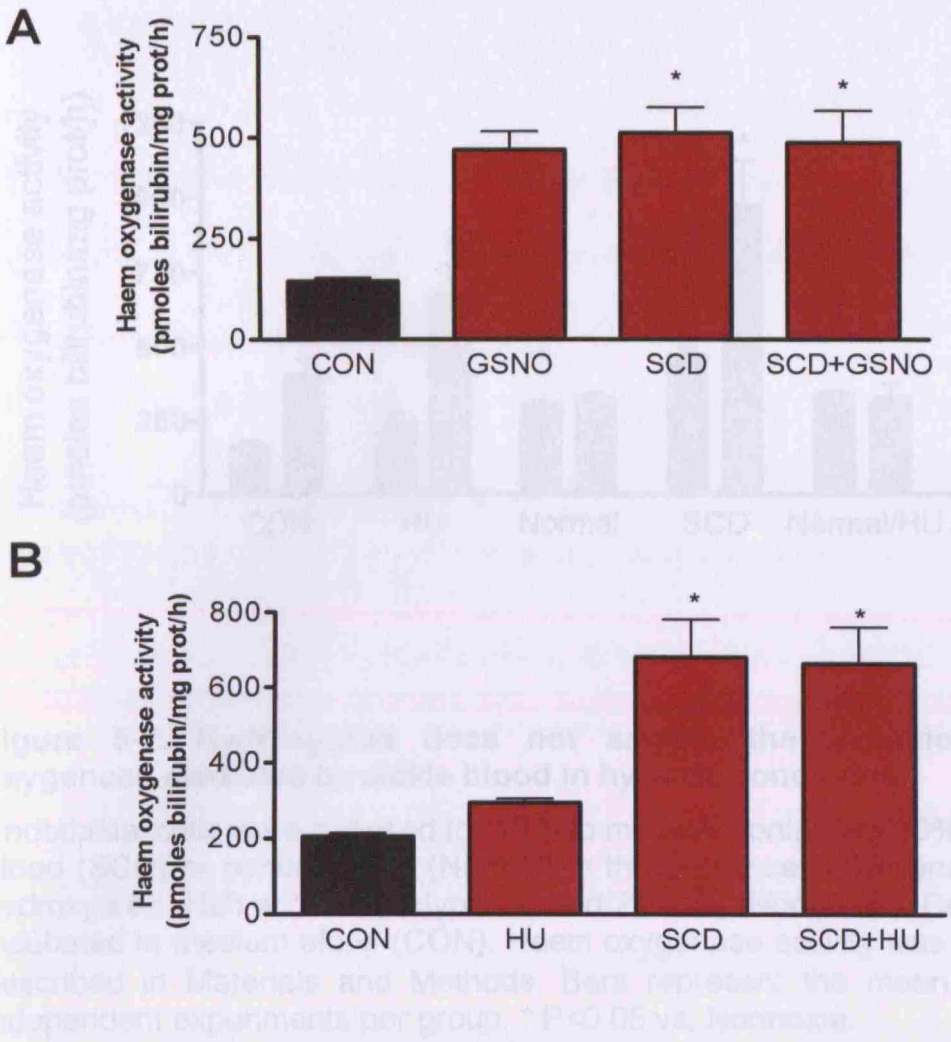


Figure 5-6. Co-incubation of NO donors with sickle blood does not affect haem oxygenase induction in endothelial cells

Endothelial cells were exposed to (A) medium containing 20% whole sickle blood in the presence or absence of 250 μM S-nitrosoglutathione (GSNO) for 6 h or (B) medium containing 20% whole sickle blood in the presence or absence of 300 μM hydroxyurea (HU) for 18 h. Cells were also incubated in medium alone (CON). Haem oxygenase activity was measured as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. CON.

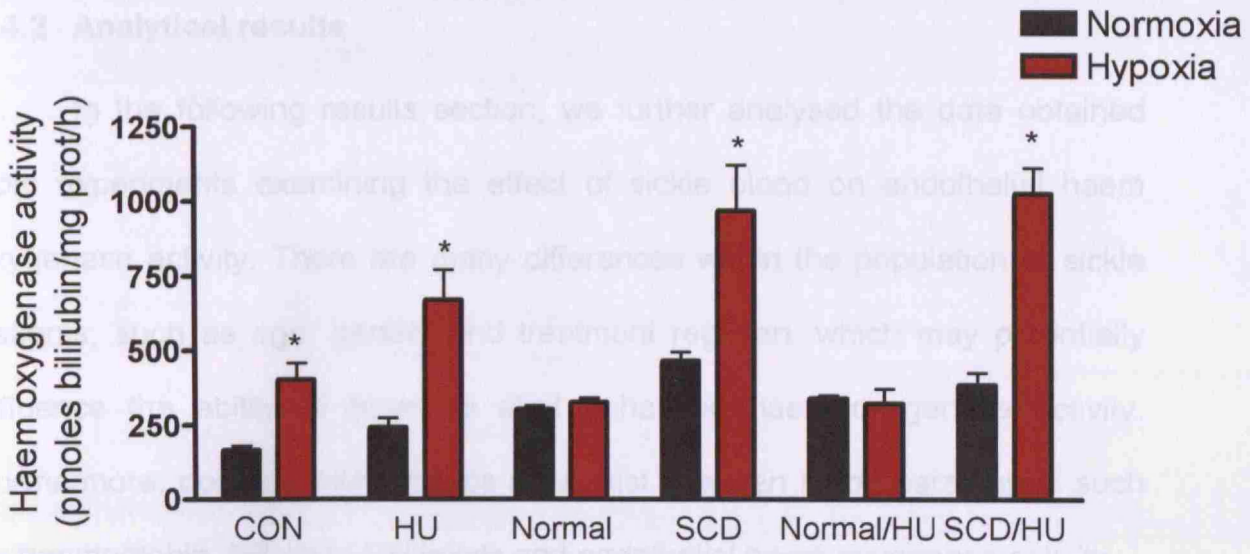


Figure 5-7. Hydroxyurea does not amplify the induction of haem oxygenase mediated by sickle blood in hypoxic conditions

Endothelial cells were exposed for 18 h to medium containing 20% whole sickle blood (SCD) or control blood (Normal) in the presence or absence of 300 μM hydroxyurea (HU) at 1% O_2 (Hypoxia) and 21% O_2 (Normoxia). Cells were also incubated in medium alone (CON). Haem oxygenase activity was measured as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. Normoxia.

5.4.2 Analytical results

In the following results section, we further analysed the data obtained from experiments examining the effect of sickle blood on endothelial haem oxygenase activity. There are many differences within the population of sickle patients, such as age, gender and treatment regimen, which may potentially influence the ability of blood to elicit enhanced haem oxygenase activity. Furthermore, possible relationships may exist between blood parameters such as haemoglobin, BR and LDH levels and endothelial haem oxygenase activity.

5.4.2.1 Induction of endothelial haem oxygenase by sickle blood can be modulated by treatment regimen

Blood taken from sickle patients, either receiving or not receiving hydroxyurea treatment, elicited significantly higher endothelial haem oxygenase activity than blood taken from control subjects. However, blood collected from patients receiving hydroxyurea treatment, elicited a lower endothelial haem oxygenase activity when compared to patients not following the drug regimen, although the difference was not statistically significant ($p=0.1547$) (Figure 5-8 A). Analysis of sickle blood showed that haemoglobin levels were similar in patients receiving or not receiving hydroxyurea treatment (Table 5-3). Additionally, sickle blood LDH and BR levels did not differ between patients under hydroxyurea regimen or not receiving treatment.

Table 5-3. Patient characteristics separated by hydroxyurea treatment

	CON (n=20)	SCD + HU (n=22)	SCD –HU (n=88)	*P†*	*P‡*	*P§*
Hb,mg/dl, mean±SD	13.7±1.07	10.28±2.0	9.88±2.2	0.0114	0.0041	0.4464
BR,μM, mean±SD	7.82±2.7	52.73±46.5	52.69±47.1	0.0106	0.0079	0.9972
LDH, Units/l, mean±SD	189±71.21	374±157	407±194	0.0311	0.0291	0.496

P† value is for the comparison of sickle patients receiving HU (SCD + HU) versus control subjects (CON).
P‡ value is for the comparison of sickle patients not receiving HU (SCD - HU) versus control subjects (CON).
P§ value is for the comparison of sickle patients receiving HU (SCD + HU) versus patients not receiving HU (SCD-HU).

In analysing whether transfusion therapy affected endothelial haem oxygenase responses, we observed that blood from sickle patients receiving transfusion regimens caused a lower endothelial haem oxygenase activity than that from patients free from transfusion; however, this difference was not statistically significant (p=0.5345) (Figure 5-8 B). Haemoglobin in blood from sickle patients receiving transfusion was not different from that measured in patients not receiving transfusion therapy (Table 5-4). Similarly, transfusion therapy did not affect blood LDH and BR levels in our sickle population. However, we note that only 5 patients from the total sickle population were receiving transfusion. Therefore, the non-significant trend of reduced serum BR and LDH in transfused patients compared to subjects not receiving transfusion could become significant if a larger number of transfused patients could be recruited and assessed.

Table 5-4. Patient characteristics separated by transfusion regimen

	CON (n=20)	SCD + Transfusion (n=5)	SCD – Transfusion (n=105)	*P†*	*P‡*	*P§*
Hb,mg/dl, mean±SD	13.7±1.07	9.6±1.3	9.98±2.2	0.0027	0.0051	0.7156
BR,μM, mean±SD	7.82±2.7	37.8±25.2	52.7±47.4	0.0051	0.0079	0.4879
LDH,U/l, mean±SD	189±71.21	313±73	402±190	0.0374	0.0282	0.3013

P† value is for the comparison of sickle patients receiving transfusion (SCD + transfusion) versus control subjects (CON).
P‡ value is for the comparison of sickle patients not receiving transfusion (SCD - transfusion) versus control subjects (CON).
P§ value is for the comparison of sickle patients receiving transfusion (SCD + transfusion) versus patients not receiving transfusion (SCD-transfusion).

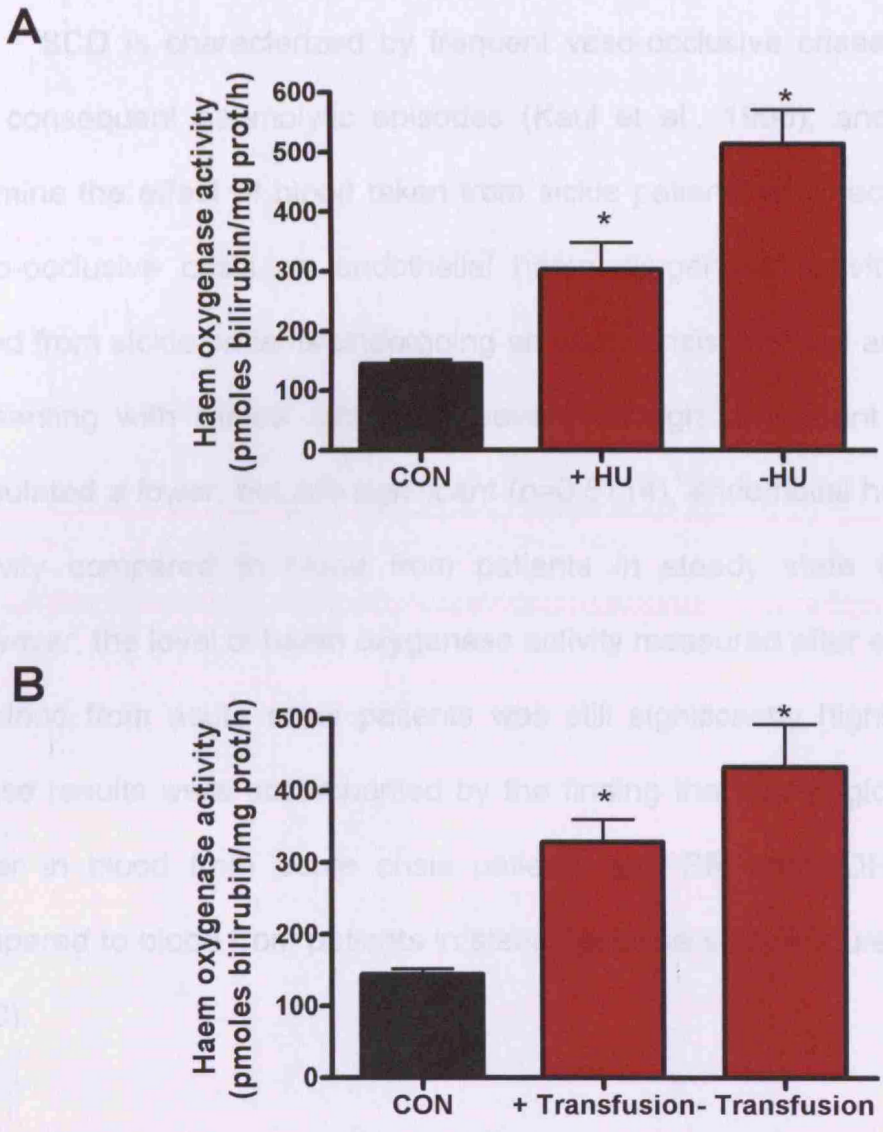


Figure 5-8. Patient treatment regimen modulates haem oxygenase induction

Endothelial cells were exposed for 6 h to (A) medium containing 20% whole sickle blood from patients receiving (+HU) or not receiving (-HU) hydroxyurea treatment and (B) medium containing 20% whole sickle blood from patients receiving transfusion (+Transfusion) or not (-Transfusion). Cells were also incubated in medium alone (CON). Haem oxygenase activity was measured as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. CON.

5.4.2.2 Induction of haem oxygenase by sickle blood is dependent on time elapsed since last sickle crisis

SCD is characterized by frequent vaso-occlusive crises in which there are consequent haemolytic episodes (Kaul et al., 1996), and we wished to examine the effect of blood taken from sickle patients who recently suffered a vaso-occlusive crisis on endothelial haem oxygenase activity. Interestingly, blood from sickle patients undergoing an acute crisis (defined as sickle patients presenting with clinical symptoms severe enough to warrant hospitalisation) stimulated a lower, but not significant ($p=0.5114$), endothelial haem oxygenase activity compared to blood from patients in steady state (Figure 5-9 A). However, the level of haem oxygenase activity measured after exposure of cells to blood from acute crisis patients was still significantly higher than control. These results were accompanied by the finding that haemoglobin levels were lower in blood from acute crisis patients and BR and LDH levels higher, compared to blood from patients in steady disease state (Figure 5-9 and Figure 5-10).

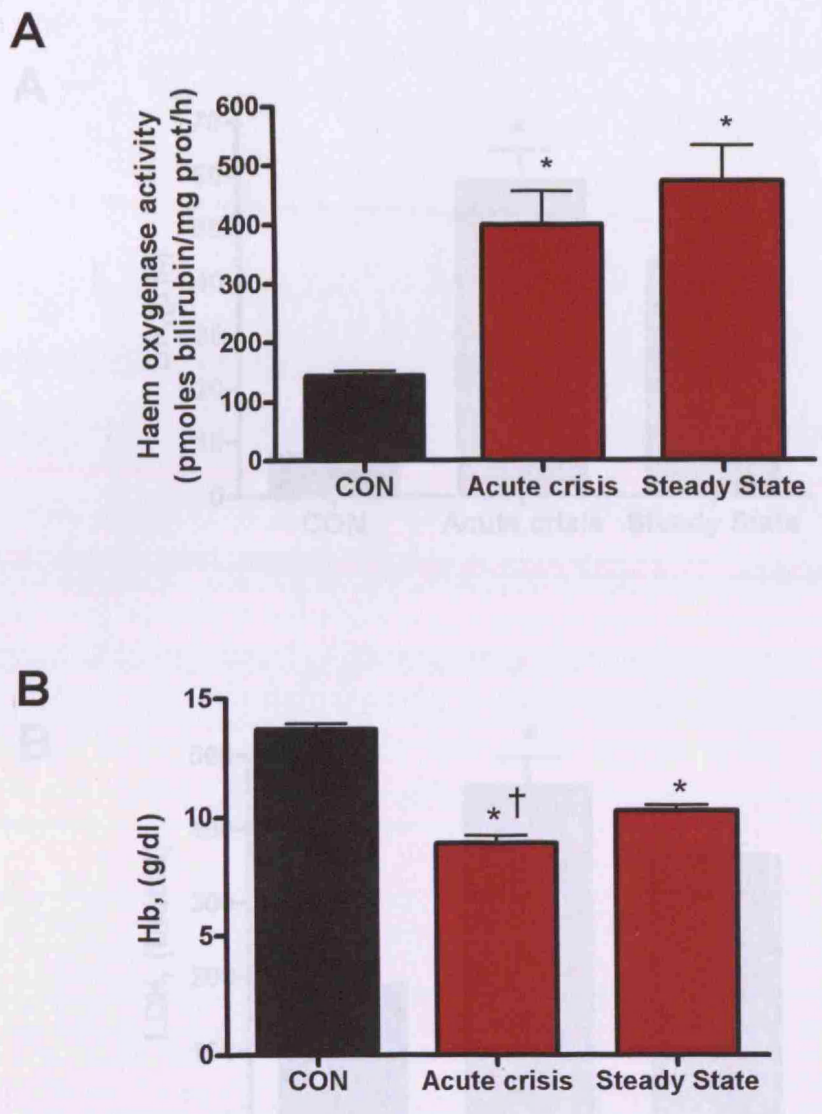


Figure 5-9. Disease state influences endothelial haem oxygenase activity and haemoglobin levels

(A) Endothelial cells were exposed for 6 h to medium containing 20% whole sickle blood taken from patients who were experiencing current acute crisis or at steady state SCD. Cells were also incubated in medium alone (CON). Haem oxygenase activity was measured as described in Materials and Methods. (B) Blood from control subjects and patients who were in acute crisis or at steady state SCD was collected and haemoglobin measurements were made as described in Materials and Methods. Bars represent the mean±SEM of 4-6 samples per group. * $P<0.05$ vs. CON or † $P<0.05$ vs. Steady state.

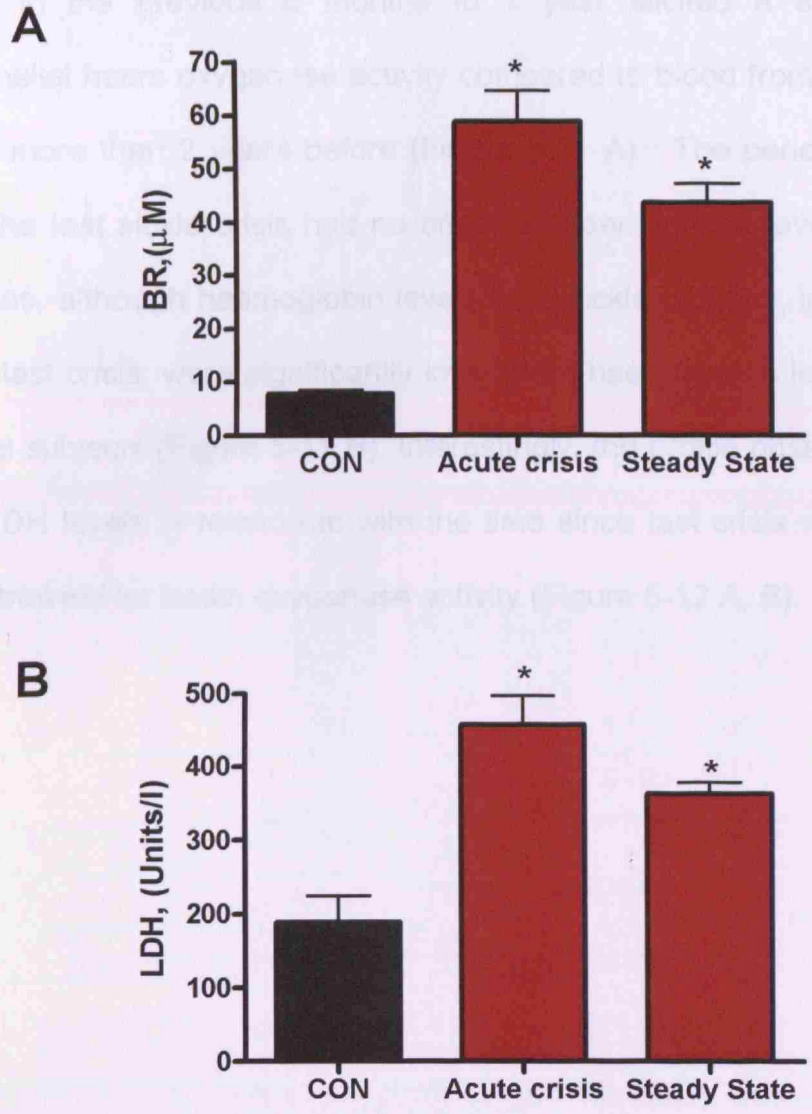


Figure 5-10. BR and LDH levels in blood of patients vary according to disease state

Blood from control subjects (CON) and sickle patients who were in acute crisis or at steady state SCD was collected and BR (A) and LDH (B) measurements were performed as described in Materials and Methods. Bars represent the mean±SEM of 4-6 samples per group.* $P<0.05$ vs. CON.

A very interesting picture emerged when the levels of haem oxygenase activity were grouped according to the time of the last crisis in the patient population. The results showed that blood from sickle patients who experienced crises in the previous 6 months to 1 year elicited a significantly higher endothelial haem oxygenase activity compared to blood from patients suffering crises more than 2 years before (Figure 5-11 A). The period of time elapsed from the last sickle crisis had no effect on haemoglobin levels in sickle blood samples, although haemoglobin levels from sickle patients, irrespective of time since last crisis, were significantly lower than haemoglobin levels in blood from control subjects (Figure 5-11 B). Interestingly, the profile observed for blood BR and LDH levels in relation to with the time since last crisis was very similar to that obtained for haem oxygenase activity (Figure 5-12 A, B).

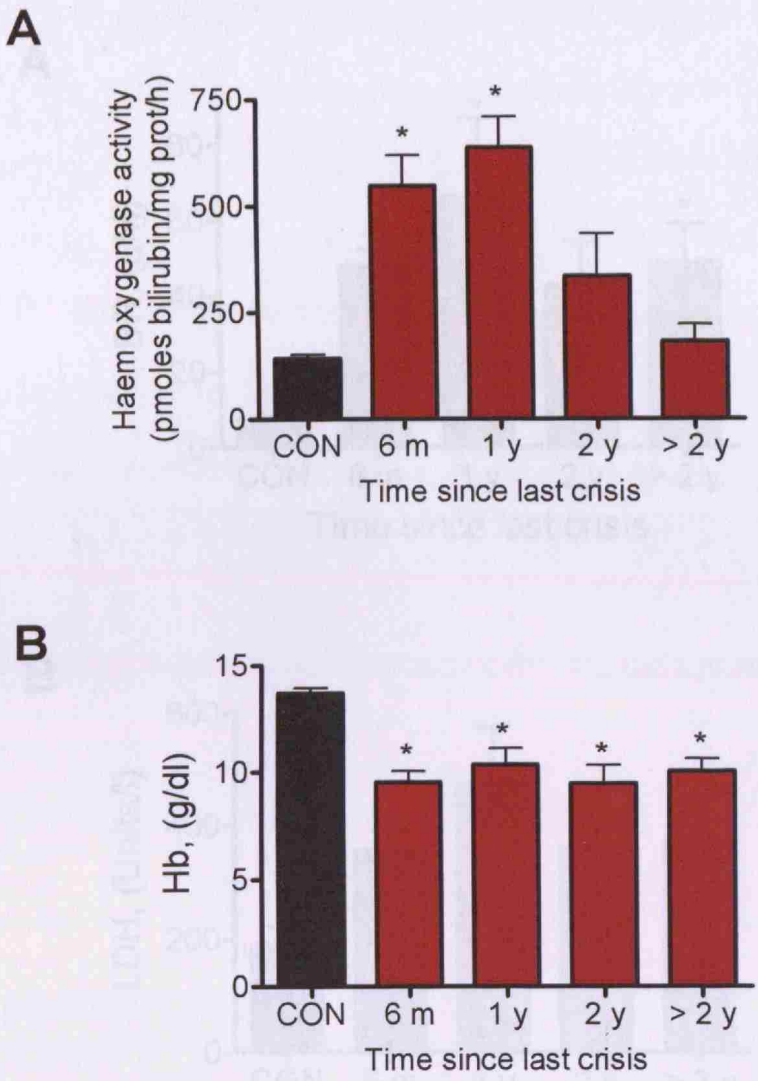


Figure 5-11. The time elapsed since the last vaso-occlusive crisis influences haem oxygenase activity levels

(A) Endothelial cells were exposed for 6 h to medium containing 20% whole sickle blood collected from patients who experienced sickle crises within the previous 6 months (6 m); 6 months to a year (1 y); 1-2 years (2 y) and greater than 2 years (>2 yr). Cells were also incubated in medium alone (CON). Haem oxygenase activity was measured as described in Materials and Methods. (B) Blood from control subjects (CON) or patients who suffered sickle crises within the previous 6 months (6 m); 6 months to a year (1 y); 1-2 years (2 y) and greater than 2 years (>2 y) was collected and haemoglobin measurements were performed as described in Materials and Methods. Bars represent the mean±SEM of 4-6 samples per group. *P<0.05 vs. CON.

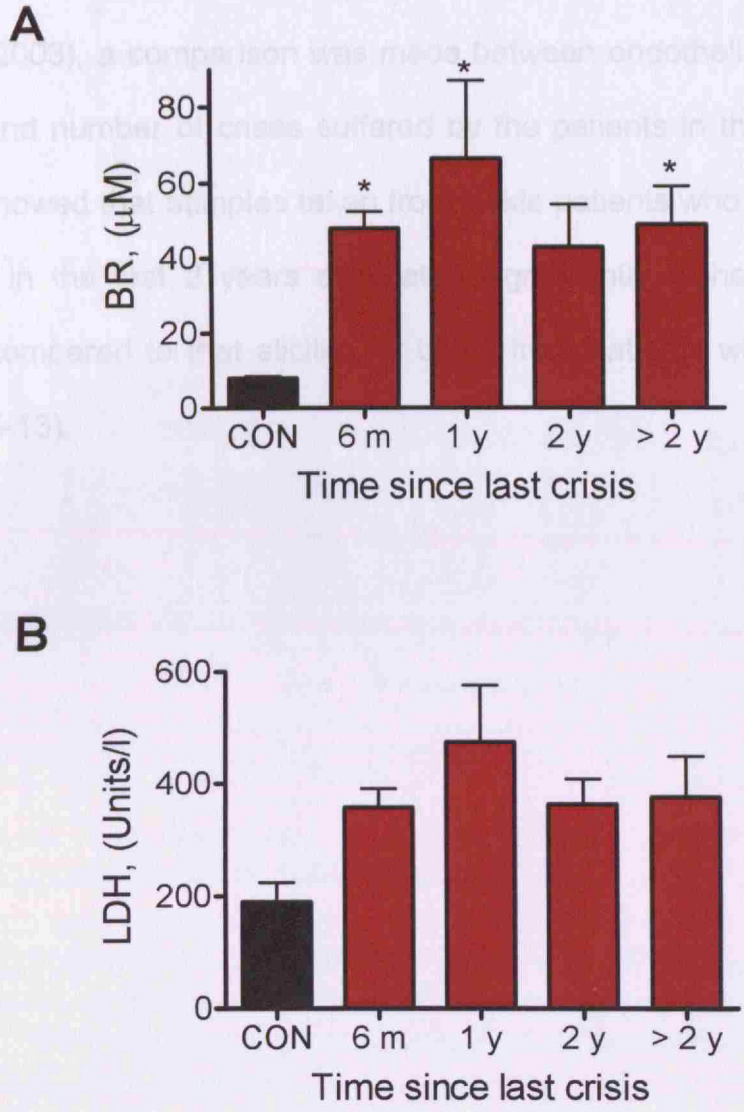


Figure 5-12. The time elapsed since the last vaso-occlusive crisis influences BR and LDH levels in blood

Blood from control subjects (CON) or patients who experienced sickle crises within the last 6 months (6 m); 6 months to a year (1 y); 1-2 years (2 y) and greater than 2 years (>2 y) was collected and BR (A) and LDH (B) measurements were performed as described in Materials and Methods. Bars represent the mean±SEM of 4-6 samples per group. * $P<0.05$ vs. CON.

As the average number of crises per year for a sickle patient is less than 1 (0.8), and a higher rate of crises increases mortality in SCD (Marchant and Walker, 2003), a comparison was made between endothelial haem oxygenase activity and number of crises suffered by the patients in the last 2 years. The results showed that samples taken from sickle patients who had experienced 1-2 crises in the last 2 years stimulated significantly higher haem oxygenase activity compared to that elicited by blood from patients who had more crises (Figure 5-13).

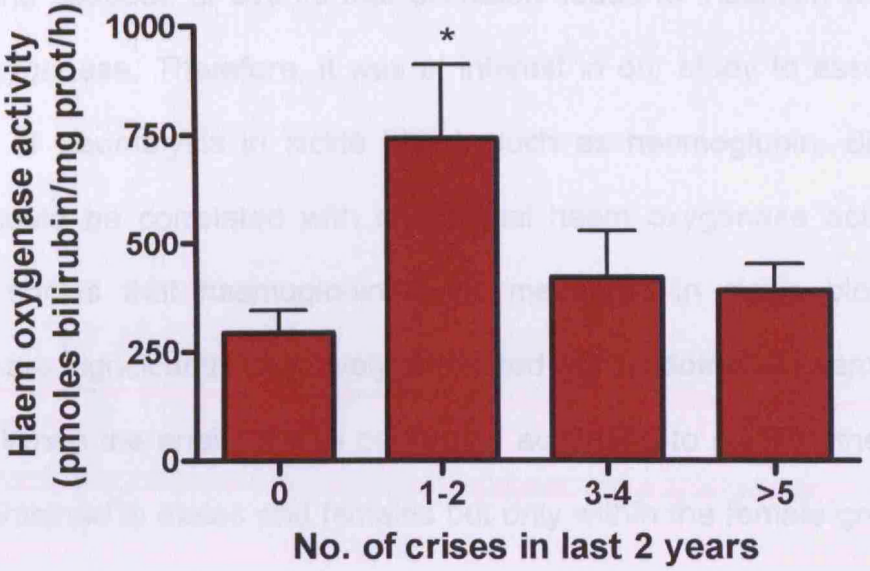


Figure 5-13. The frequency of sickle crises influences and endothelial haem oxygenase activity stimulated by blood

Endothelial cells were exposed for 6 h to medium containing 20% whole sickle blood from patients who suffered 0, 1-2, 3-4 or >5 sickle crises in the last 2 years. Haem oxygenase activity was measured as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. *P<0.05 vs. No crises (0).

5.4.2.3 Haem oxygenase activity is correlated with haemoglobin, LDH, BR level and age and gender

Haemolysis is prevalent in SCD and could be postulated to be the first step in the cascade of events that ultimately leads to induction of endothelial haem oxygenase. Therefore, it was of interest in our study to assess whether markers of haemolysis in sickle blood, such as haemoglobin, BR and LDH levels, could be correlated with endothelial haem oxygenase activity. Figure 5-14 A shows that haemoglobin levels measured in sickle blood from all patients are significantly negatively correlated with endothelial haem oxygenase activity. When the analysis was performed according to gender, the correlation was maintained in males and females but only within the female group was the correlation still significant (Figure 5-14 B). Sickle blood BR levels from all patients were positively and significantly correlated with haem oxygenase activity (Figure 5-15 A). However, the analysis carried out according to gender showed that the correlation between haem oxygenase activity and BR was strong and significant for female patients, while for males no meaningful correlation was detected (Figure 5-15 B).

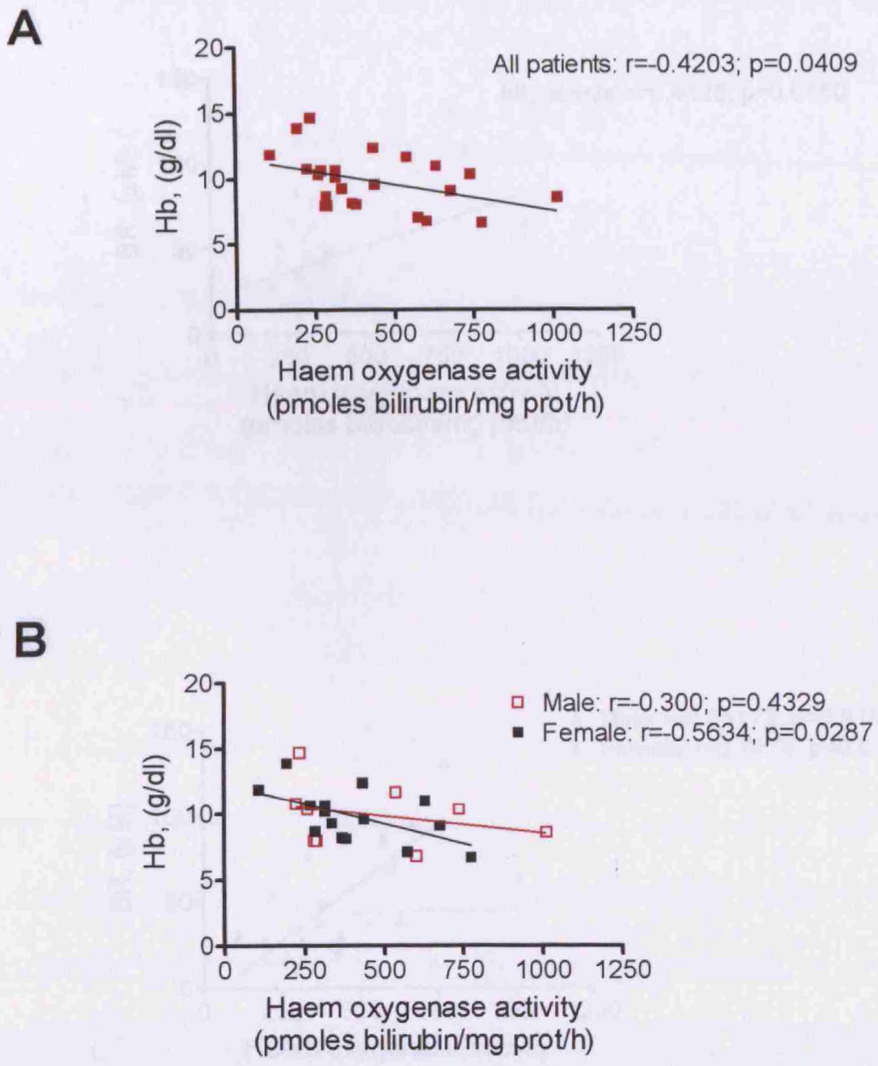


Figure 5-14. Correlation between endothelial haem oxygenase activity and haemoglobin levels

The scatter graph illustrates the relationship between endothelial haem oxygenase activity stimulated by sickle blood and haemoglobin levels in all sickle patients (■) (A) and male (□) or female (■) sickle patients (B). Haem oxygenase activity and measurement of blood haemoglobin were performed as described in Materials and Methods. r = Pearson's correlation.

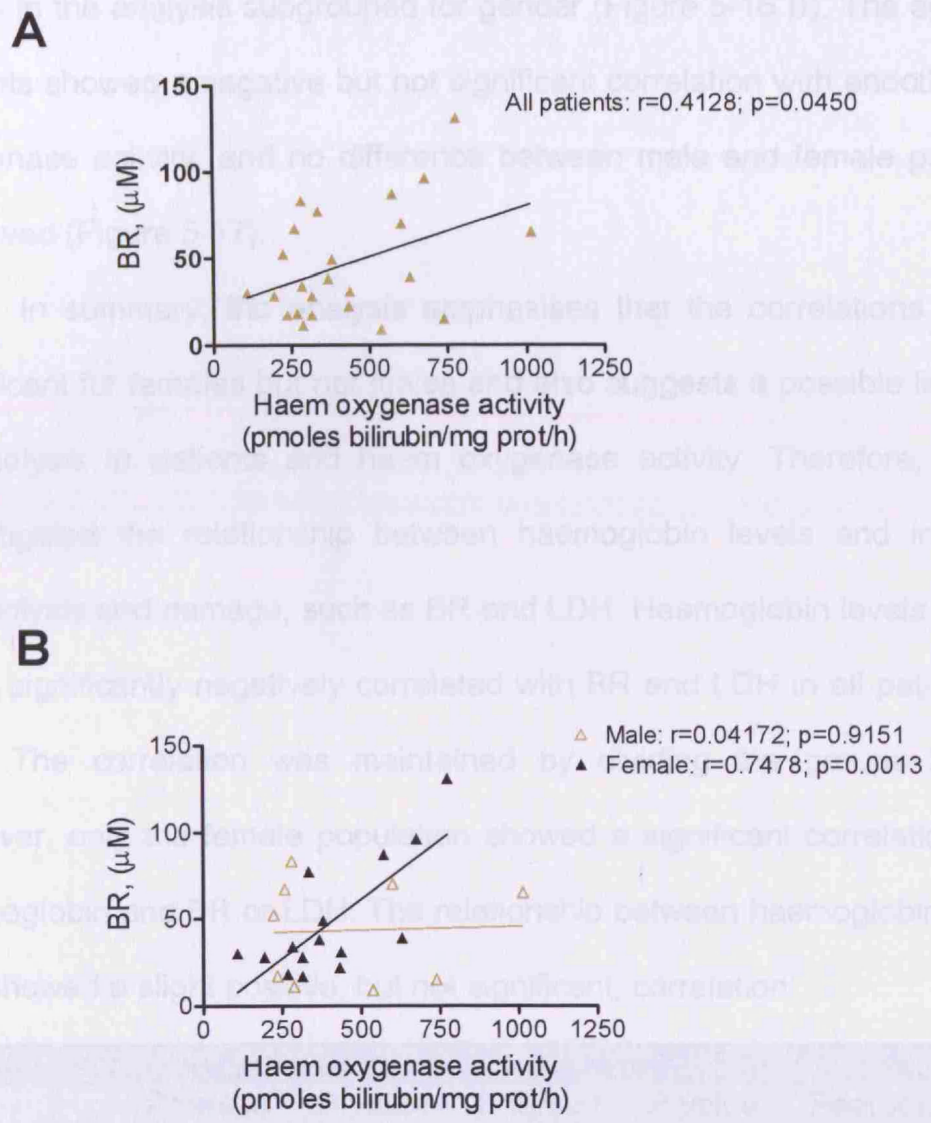


Figure 5-15. Correlation between haem oxygenase activity and BR levels

The scatter graph illustrates the relationship between endothelial haem oxygenase activity stimulated by sickle blood and BR levels in all sickle patients (▲) (A) and male (△) or female (▲) sickle patients (B). Haem oxygenase activity and measurement of blood BR were performed as described in Materials and Methods. r = Pearson's correlation.

A non-significant positive correlation was also observed between haem oxygenase activity and LDH levels in all patients (Figure 5-16 A). Again, the correlation emerged strong and significant for female patients but was lost in males in the analysis subgrouped for gender (Figure 5-16 B). The age of sickle patients showed a negative but not significant correlation with endothelial haem oxygenase activity, and no difference between male and female patients was observed (Figure 5-17).

In summary, the analysis emphasises that the correlations are mainly significant for females but not males and also suggests a possible link between haemolysis in patients and haem oxygenase activity. Therefore, we further investigated the relationship between haemoglobin levels and indicators of haemolysis and damage, such as BR and LDH. Haemoglobin levels were found to be significantly negatively correlated with BR and LDH in all patients (Table 5-5). The correlation was maintained by dividing the groups by gender; however, only the female population showed a significant correlation between haemoglobin and BR or LDH. The relationship between haemoglobin levels and age showed a slight positive, but not significant, correlation.

Parameters	All patients Hb		Female Hb		Male Hb	
	Pearson r	P value	Pearson r	P value	Pearson r	P value
BR	-0.6188	0.0013	-0.6804	0.0052	-0.5371	0.1360
LDH	-0.498	0.0205	-0.5254	0.0443	-0.4261	0.2528
Age	0.1773	0.4073	0.2154	0.4407	0.1285	0.7419

Table 5-5. Pearson correlations between haemoglobin levels in male or female patients and BR, LDH levels or age

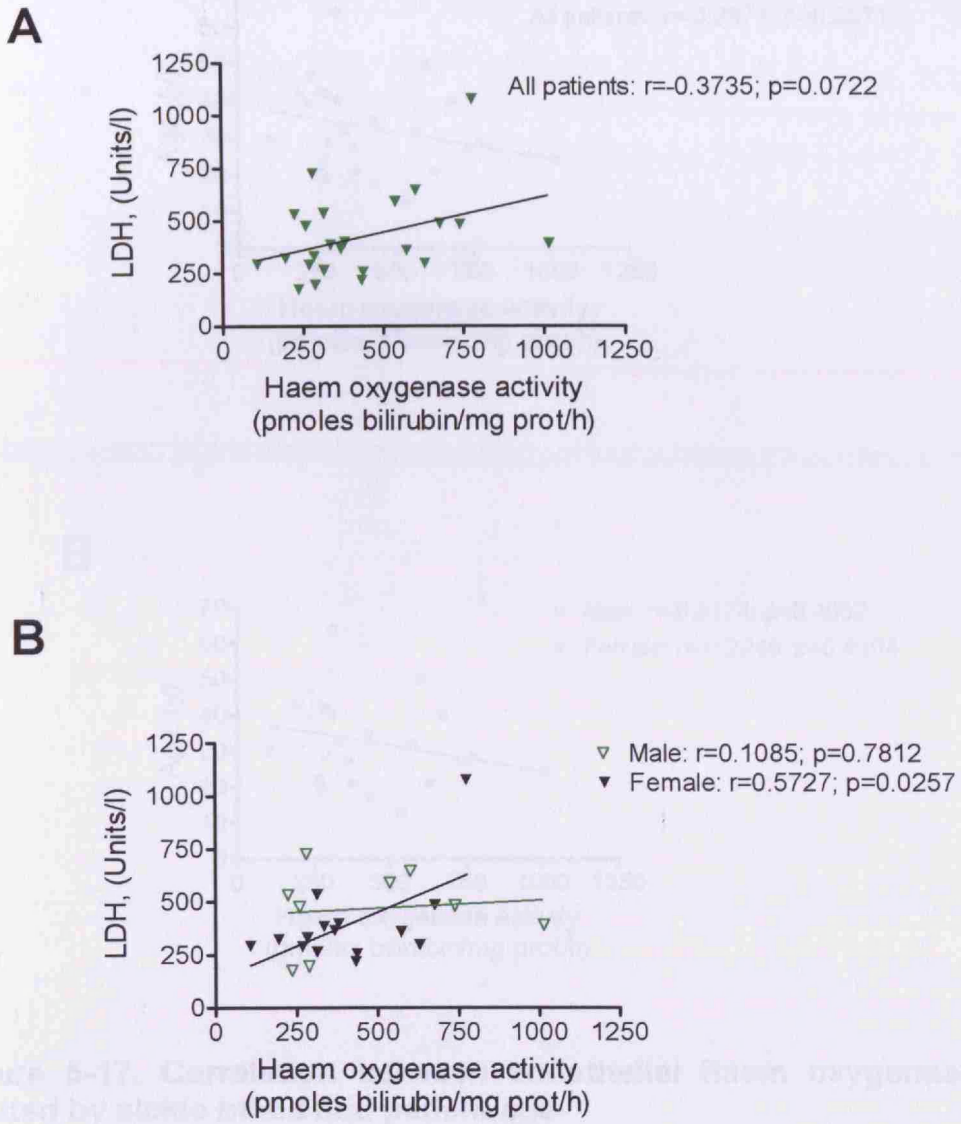


Figure 5-16. Correlation between endothelial haem oxygenase activity elicited by sickle blood and LDH levels

The scatter graph illustrates the relationship between endothelial haem oxygenase activity stimulated by sickle blood and LDH levels in all sickle patients (▼) (A) and male (▽) or female (▼) sickle patients (B). Haem oxygenase activity and measurement of blood LDH were performed as described in Materials and Methods. r = Pearson's correlation.

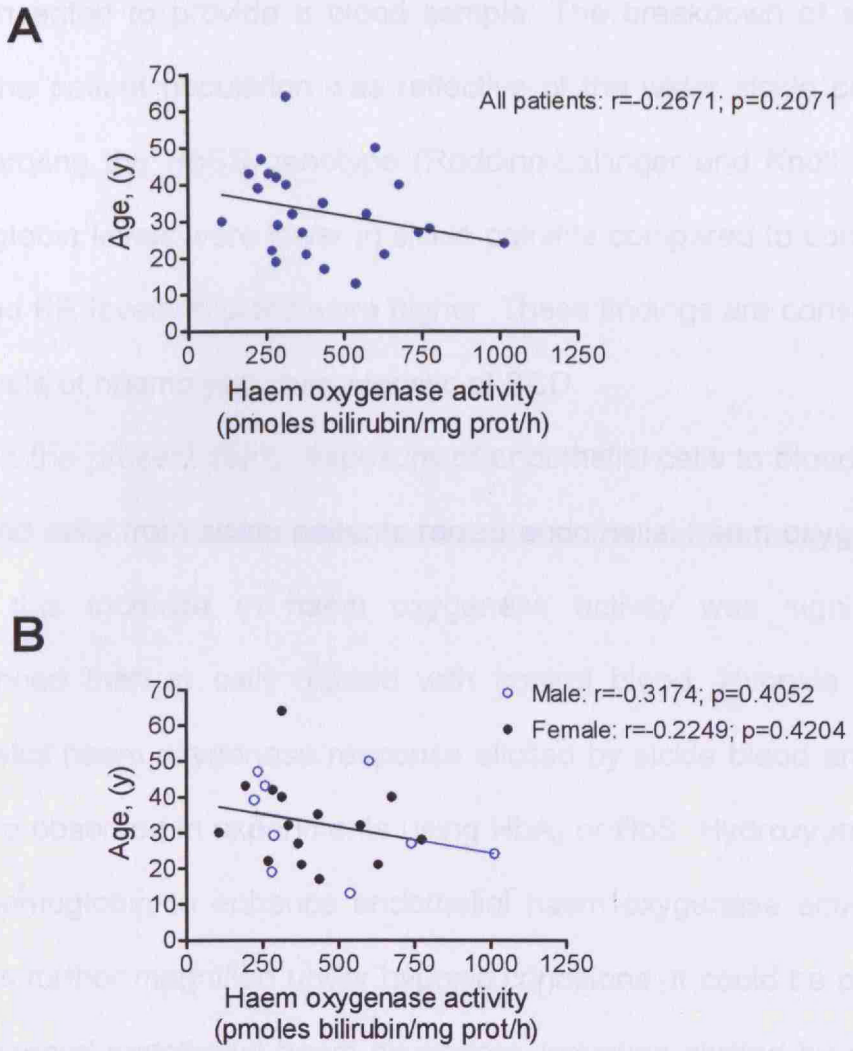


Figure 5-17. Correlation between endothelial haem oxygenase activity elicited by sickle blood and patient age

The scatter graph illustrates the relationship between endothelial haem oxygenase activity stimulated by sickle blood and age of all sickle patients (●) (A) and male (○) or female (●) sickle patients (B). Haem oxygenase activity measurement was performed as described in Materials and Methods. r = Pearson's correlation.

5.5 Discussion

One hundred and ten patients with SCD were recruited for this study, and consented to provide a blood sample. The breakdown of sickle variants within the patient population was reflective of the wider sickle population with 71% carrying the HbSS genotype (Redding-Lallinger and Knoll, 2006). Blood haemoglobin levels were lower in sickle patients compared to controls whereas LDH and BR levels in blood were higher. These findings are consistent with the high levels of haemolysis characteristic of SCD.

In the present study, exposure of endothelial cells to blood or separated red blood cells from sickle patients raised endothelial haem oxygenase activity levels; this increase in haem oxygenase activity was significantly more pronounced than in cells treated with control blood. Hypoxia amplified the endothelial haem oxygenase response elicited by sickle blood and this pattern was also observed in experiments using HbA_0 or HbS. Hydroxyurea synergised with haemoglobin to enhance endothelial haem oxygenase activity, an effect that was further magnified under hypoxic conditions. It could be postulated that the enhanced endothelial haem oxygenase induction elicited by sickle blood is an important protective mechanism against oxidative stress caused by haemolytic events, and the observation that hydroxyurea enhances haem oxygenase activity in the presence of haemoglobin suggests an additional mechanism of therapeutic action for this drug.

The observation that incubation of endothelial cells with plasma from sickle patients did not induce haem oxygenase activity is interesting. As well as cell-free haemoglobin, they are many inflammatory mediators within the plasma that could have potentially increased haem oxygenase. It is possible that 10%

plasma is insufficient to elicit a haem oxygenase response and higher concentrations may be required for this purpose. Conversely, plasma components with anti-oxidant properties may prevent a rise in haem oxygenase activity. For example, plasma is rich in albumin, which exerts anti-oxidant properties (Cha and Kim, 1996) and binds NO (Orie et al., 2005), a potent inducer of HO-1. Interesting follow up studies could investigate different plasma fractions for their anti-oxidant or NO scavenging effects. Indeed, the plasma constituent S-nitrosoalbumin has been found to protective in an animal model of SCD (De Franceschi et al., 2006).

The release of free haemoglobin from ruptured red blood cells could be hypothesised as the trigger for the raised haem oxygenase activity in endothelial cells exposed to sickle blood. Indeed, haem released from haemoglobin is uptaken by endothelial cells to induce HO-1 and ferritin, both of which have been widely reported to protect the endothelium from oxidative damage (Balla et al., 1992b). Of interest, induction of HO-1 has been reported in circulating endothelial cells (Nath et al., 2001a) from sickle patients. Circulating endothelial cells are found in very low numbers physiologically, but are raised considerably in SCD. The enhanced HO-1 activity observed in these circulating cells has been postulated to offer a protective response to a pro-inflammatory environment (Solovey et al., 1997), and it could be inferred from these studies and our findings that raised HO-1 levels in endothelial cells *in situ* could have a similar protective role in SCD. The results showing no effect of sickle plasma on haem oxygenase are surprising, since haem levels can reach up to 20 μM in sickle plasma (Reiter et al., 2002) and inflammatory mediators capable of stimulating haem oxygenase may be present.

Hypoxia is the trigger for sickling and haemolysis in SCD, as it is the deoxygenation of HbS that results in polymerization of the mutant haemoglobin, increasing the susceptibility of red blood cells to sickle, damage cellular membranes and eventually rupture (Bunn, 1997). Our observation that hypoxia enhanced the haem oxygenase activity elicited by sickle blood may be explained by the increased haemolysis triggered by hypoxia. Hypoxia is also capable of inducing endothelial haem oxygenase independently (Motterlini et al., 2000) and up-regulation of the protein in SCD could protect cells against further hypoxic endothelial damage, such as the expression of adhesion molecules and release of potent vasoconstrictors (Kourembanas, 2002), which may both contribute to vaso-occlusive events.

It is important to clarify the terms normoxia and hypoxia in regards to our experimental protocol. For cell culture protocols we define normoxia and hypoxia as 21% and 1% O_2, respectively. However, physiological *in vivo* levels of O_2 are normally much lower with blood carrying the equivalent of 10-12.5% O_2 and healthy tissue having levels of 3-6% O_2 (Atkuri et al., 2005). Therefore, standard culture conditions (21% O_2) expose cells to 2 to 5-fold higher oxygen conditions than would be found *in vivo* and the reactions between O_2, NO and haem we observed under our *in vitro* conditions may not be representative of the *in vivo* situation. This problem could be overcome by bubbling haemoglobin or blood solutions with O_2 until physiological oxygen levels are reached before incubation with cells. Similarly, although 1% oxygen is well below physiological O_2 levels, it may not represent accurately the intermittent regional hypoxia prevalent in sickle cell disease and an *in vivo* sickle model, e.g., transgenic sickle mouse, would more closely emulate the pathophysiological state.

As shown in this chapter, the ability of hydroxyurea to induce haem oxygenase activity in endothelial cells was significantly increased under hypoxic conditions. In addition, co-incubation of hydroxyurea with HbA_0 or HbS significantly enhanced the haem oxygenase activity elicited by these haemoglobins. The observed synergism between haemoglobin and hydroxyurea could be a result of the NO donor properties of the drug, as reported earlier (King, 2004); for example, NO released from hydroxyurea binds haemoglobin, allowing for easier uptake by the endothelial cell and subsequent induction of haem oxygenase (Foresti et al., 2003; Foresti et al., 2006). Our data on the release of NO from hydroxyurea only shows a very slight increase in detectable NO when hydroxyurea is incubated with HbS (see chapter 3), suggesting that more experiments are required to prove this hypothesis. Therefore, the mechanism of therapeutic action of hydroxyurea in SCD could involve a number of components. On one side, long-term intake of hydroxyurea increases HbF production, thus reducing the incidence of crises and haemolysis. Indeed, we observed a lower endothelial haem oxygenase induction with blood taken from sickle patients on hydroxyurea compared to those on no treatment, which could possibly reflect a lower free haemoglobin level due to reduction in haemolysis. In addition, haemoglobin from a haemolytic event may synergise with hydroxyurea during acute situations to induce haem oxygenase activity and provide protection from the damaging effects of free haem. However, our data with sickle blood and hydroxyurea (Figure 5-6) did not support this hypothesis. An explanation for these results could be limitations in our experimental protocol. For example, scavenging of hydroxyurea could be occurring by blood components which may thus limit its

pharmacological activity and possibly limit interactions with haemoglobin and subsequent effects on haem oxygenase activity. Recent studies sustain the idea that hydroxyurea might work by mechanisms unrelated to HbF synthesis, such as reduction in neutrophil levels, modulation of adhesion molecule expression in erythrocytes and erythropoietin production (Steinberg and Brugnara, 2003).

Our population included patients on hydroxyurea, transfusion or no treatment regimens and there were also differences in the population with regards to the number of sickle crises suffered and time elapsed from the last crisis. As all of these factors could affect the induction of haem oxygenase, an analysis of the relationship between patient characteristics and haem oxygenase activity was required.

Within the sickle population, the average rate of crisis is 0.8 per year and greater numbers of crisis per year are an indicator of more severe disease and increased mortality (Marchant and Walker, 2003). Intriguingly, our results show that blood from patients who experienced a crisis in the previous year stimulated enhanced endothelial haem oxygenase levels compared to those who were crisis-free for 2 or more years. It may be that haem oxygenase levels were induced by blood components released during the haemolytic crisis and that these components remained present for a certain extended period after the crisis. Interestingly, recent studies have indicated that clinical manifestations in SCD may be the result of a chronic inflammatory state secondary to abnormal erythrocyte membranes and haemolysis (Chies and Nardi, 2001; Frenette and Atweh, 2007). As HO-1 is induced by inflammatory mediators (Otterbein et al.,

2003a), our results could indicate that enhanced levels of endothelial haem oxygenase are caused by the inflammation produced by a sickle crisis.

Intriguingly, blood from sickle patients who had 1-2 crises in the previous 2 years stimulated much higher endothelial haem oxygenase levels than blood from patients who had more crises. Since higher crisis rates are linked with increased mortality in SCD and a higher number of crises is associated with lower levels of haem oxygenase activity, we propose that these patients could represent a subset of SCD sufferers who lack the ability to induce this protective enzyme and are therefore susceptible to more crises. In the clinical setting, the problem may be compounded by repeated haemolytic episodes overwhelming endothelial haem oxygenase and causing irreversible changes to the endothelium.

Interestingly, sickle blood from patients in acute crisis elicited a lower endothelial haem oxygenase response than blood from patients in a steady disease state. These results would seem contradictory to the proposed hypothesis that haemolytic crisis would raise haem oxygenase levels. However, the levels of haem oxygenase activity seen after exposure of cells to blood from acute crisis patients is still significantly higher than blood from control subjects. A possible explanation for these findings could be that an acute haemolytic crisis might overwhelm the haem degradation system and/or it may take a while for the haem oxygenase response to develop.

The main aim of the therapy in SCD is to reduce the sickling of red blood cells, as this is the trigger to the extensive cellular and organ damage seen in the disease. Many approaches to solve this problem have been tried, but the most effective are through blood transfusion or treatment with the drug

hydroxyurea (Redding-Lallinger and Knoll, 2006), thus reducing the number of sickled cells and consequent haemolysis. Therefore, it was not surprising that blood from patients on hydroxyurea or transfusion treatment elicited lower endothelial haem oxygenase activity when compared to blood from patients not receiving treatment. However, the endothelial haem oxygenase activity elicited by blood from patients on either treatment regimen was still higher than control blood, suggesting that haemolysis is still occurring in these patients, but at a reduced rate compared to those without treatment.

A significant correlation between markers of haemolysis in blood and stimulated endothelial haem oxygenase activity was observed. In the case of haemoglobin, levels in sickle blood were negatively correlated with haem oxygenase activity and we propose that low levels of haemoglobin might reflect higher free haemoglobin and haem levels due to increased haemolysis and lead to a subsequent increase in endothelial haem oxygenase activity. Accordingly, we also observed a positive correlation between BR or LDH levels and endothelial haem oxygenase activity. As these two blood parameters are markers indicative of red blood cell haemolysis, it is reasonable that raised levels of haemolysis would parallel enhanced levels of endothelial haem oxygenase activity. In addition, blood haemoglobin levels were also significantly negatively correlated with BR and LDH levels, confirming that low haemoglobin levels were linked with an increase in haemolysis. A correlation between haemolysis and increased vascular endothelial haem oxygenase activity has been observed in previous studies (Balla et al., 2003) and has been suggested to function as an endothelial adaptation to raised haem levels and serve as protection against haem-mediated oxidative damage. The importance of this

adaptation is further reinforced by the presence of severe endothelial damage and increased sensitivity to oxidative damage in the case of a HO-1 deficient child (Yachie et al., 1999).

The analysis of haem oxygenase endothelial responses based on gender elicited some interesting results. Female sickle patients showed significant correlations between endothelial haem oxygenase response and haemoglobin, BR and LDH levels, while male patients did not show significant correlation with any of the blood parameters examined. This is an interesting observation and demonstrates differences in the ability to stimulate haem oxygenase responses between male and female sickle patients. Indeed, differences in biological responses between male and female patients in SCD have been reported previously. For example, Gladwin and colleagues (Gladwin et al., 2003) observed that NO bioavailability and NO responsiveness are greater in women than in men with SCD. This is an intriguing observation, as NO is also a powerful inducer of haem oxygenase and its enhanced bioavailability in blood from female sickle patients could explain the stronger positive correlation observed between endothelial haem oxygenase activity and markers of haemolysis (BR and LDH) compared to blood from males. Furthermore, the average life-span for sickle female patients is longer than in men and males also suffer more painful sickle crises, suggesting that females have better adaptive responses to SCD than men (Chang et al., 1995; Dover et al., 1992).

Repeated exposure of endothelium to haemolytic events could possibly affect haem oxygenase responses in SCD. Although not significant, there was a trend for the response of endothelial haem oxygenase to sickle blood to diminish as patients got older. These data show a greater haem oxygenase

response in younger patients, which could eventually diminish over time due to repeated oxidative damage to the endothelium and to a general decline in physiological functions. An age-dependent decline in induction of heat shock proteins, such as HO-1, has already been associated with increased oxidative injury in other reports (Ewing and Maines, 2006; Moore et al., 2007). Taking into account the large variability in morbidity and mortality within the sickle population we hypothesise that the lower ability of patients to physiologically respond to sickle crises is associated with higher mortality rates and that the endothelial haem oxygenase response may play a role in the phenomenon.

Figure 5-18 shows a proposed mechanism of induction and action of endothelial haem oxygenase in SCD. Chronic haemolysis and subsequent raised levels of free haemoglobin and haem would induce the haem-degrading enzyme haem oxygenase, whose induction not only would reduce the haem burden and therefore oxidative stress levels, but also release the haem breakdown products, CO and BR, which possess important bioactive properties and elicit pharmacological activities that counteract the damaging effects of free haem. CO, as well as having vasodilatory properties, has also been shown to exert anti-inflammatory, anti-apoptotic and anti-proliferative functions in various cell types, including endothelial cells (Otterbein et al., 2003a). Importantly, administration of CO gas has already been reported to have therapeutic actions in SCD. Over 30 years ago, Beutler conducted a pioneering clinical study exploring the effects of CO inhalation on the sickling of red blood cells in sickle patients (Beutler, 1975). The study demonstrated that inhalation of CO by sickle patients over a 5 day period resulted in a significant increase in the life-span of red blood cells in sickle blood, thereby reducing the susceptibility of red blood

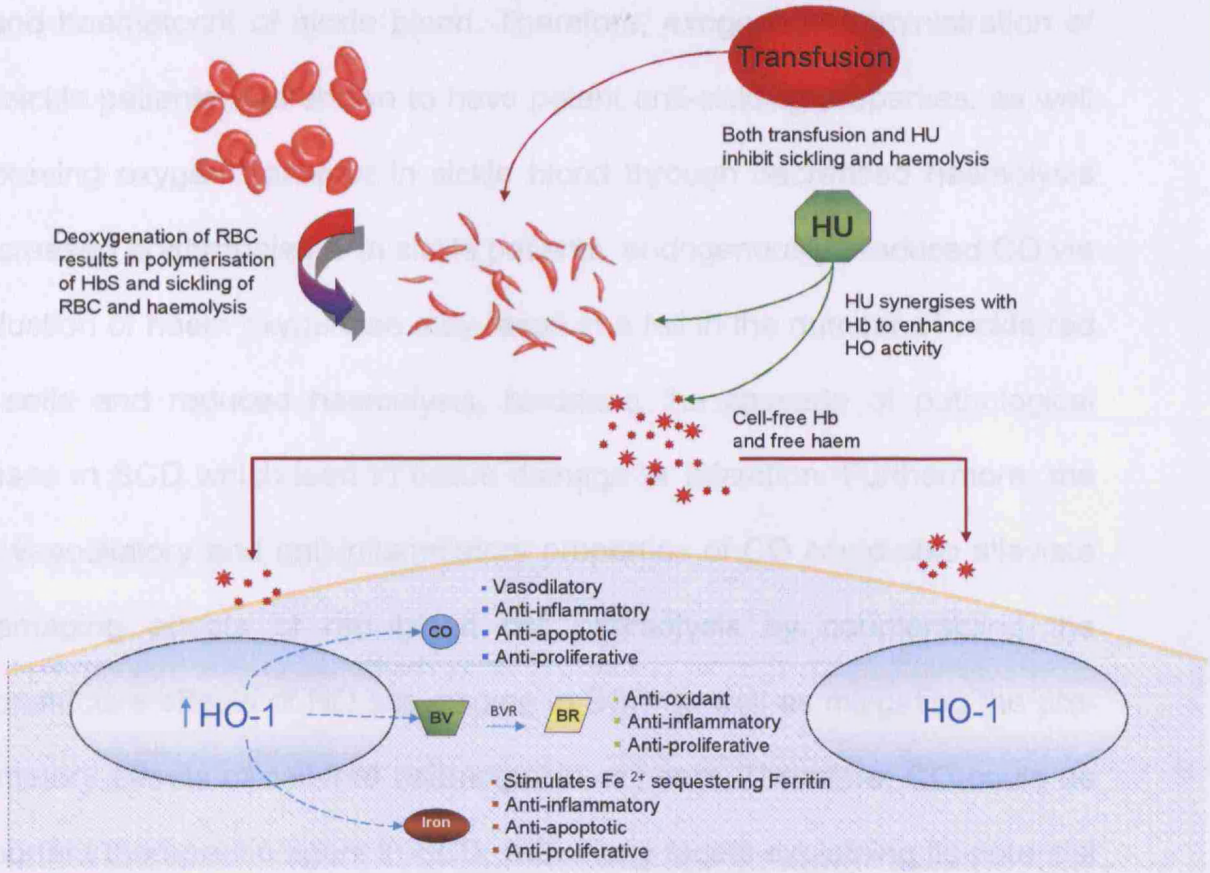


Figure 5-18. Possible mechanism of action for haem oxygenase induction after exposure of endothelium to sickle blood

The sickling of red blood cells (RBC) in SCD promotes haemolysis and the release of free haemoglobin (Hb) and haem, which can be uptaken by endothelial cells to induce haem oxygenase-1 (HO-1). HO-1 degrades haem and releases carbon monoxide (CO), biliverdin (BV) and iron (Fe^{2+}). BV is further converted into bilirubin (BR) by the action of biliverdin reductase (BVR). Transfusion therapy in SCD can diminish sickling by reducing the concentration of HbS-containing red blood cells. Hydroxyurea (HU) can also reduce the sickling of RBCs by increasing intracellular HbF production, and in addition can synergise with Hb to stimulate endothelial HO-1.

cells to sickle and subsequently rupture. The therapeutic action of CO to prevent sickling was attributed to CO binding to the HbS molecule and maintaining it in the more stable oxyhaemoglobin conformation, which hindered the polymerisation of HbS and subsequent sickling of the red blood cell. In addition to an anti-sickling action, CO was also observed to stimulate erythropoiesis in sickle patients, which significantly increased the haemoglobin

level and haematocrit of sickle blood. Therefore, exogenous administration of CO in sickle patients was shown to have potent anti-sickling properties, as well as improving oxygen transport in sickle blood through decreased haemolysis and increased erythropoiesis. In sickle patients, endogenously produced CO via the induction of haem oxygenase may result in a fall in the number of sickle red blood cells and reduced haemolysis, hindering the cascade of pathological processes in SCD which lead to tissue damage or infarction. Furthermore, the potent vasodilatory and anti-inflammatory properties of CO could also alleviate the damaging effects of red blood cell haemolysis by counteracting the vasoconstrictive effects of NO scavenging in SCD as well as mitigating the pro-inflammatory effects of cell-free haemoglobin or haem. Therefore, CO could be an important therapeutic agent in SCD, with many facets explaining its potential therapeutic action. Indeed, low dose inhalation of CO has been reported to inhibit vascular stasis and diminish adhesion molecule expression in a transgenic sickle mice model (Belcher et al., 2006), strengthening the idea that CO could have a beneficial role in SCD. However, the toxic effects of CO must also be taken into account when considering CO as a therapeutic tool. The recent development of CO-RMs, which have been shown to carry and deliver CO safely in biological systems (Alberto and Motterlini, 2007), offers a novel strategy to harness the potential therapeutic effects of CO. Indeed, CO-RMs have produced significant therapeutic effects in a variety of experimental animal models (Clark et al., 2003); (Motterlini et al., 2002a).

In SCD, BR has also been reported to have important anti-oxidant effects on sickle cell membranes by protecting them from peroxidation and therefore reducing the susceptibility of the sickle erythrocytes to rupture (Dailly et al.,

1998). Furthermore, high serum levels of BR have been correlated with vascular resistance to oxidative stress, and high BR levels are indicative of a reduced incidence of coronary artery disease. Indeed, the incidence of atherosclerotic disease in SCD patients is very low. SCD patients have low cholesterol and HDL-C levels which are thought to be due to altered metabolism of lipids (Rahimi et al., 2006). The presence of significant reduction in total cholesterol and LDL-cholesterol in SCD is thought to result in a lower prevalence of coronary artery disease. However, an increase in BR levels from haem oxygenase catalysed haem breakdown may be another mechanism by which the risk of coronary artery disease is reduced, as administration of BR has been shown to be cardio-protective in animal models of ischaemic heart injury (Clark et al., 2000b; Foresti et al., 2001). Therefore, administration of BR, on its own or in conjunction with CO, could be of therapeutic use in SCD, mirroring the beneficial effects of haem oxygenase induction. This therapeutic approach may be particularly useful for sickle patients with inherently low haem oxygenase responses.

Intriguingly, there is a huge phenotypic heterogeneity in SCD arising from a single gene mutation. Genetic polymorphisms may explain the range of clinical responses observed in this disease. Indeed, polymorphisms in coagulation, cell adhesion and cell hydration pathways are thought to influence the risk of priapism in SCD (Elliott et al., 2007). If the release of cell-free haemoglobin from ruptured red blood cells is the trigger for a cascade of deleterious effects in SCD, it could be proposed that polymorphisms in the proteins responsible for dealing with haemoglobin and haem homeostasis modulate the effect that free haem has on cellular targets. Potential

polymorphisms in the haem-catabolising enzymes HO-1 and BVR, as well as haemoglobin scavenger proteins haptoglobin and CD-163, could greatly modify individual responses to haemolytic episodes (Exner et al., 2001; Van Vlierberghe et al., 2004). Therefore, a sub-set of sickle patients may exist who have a reduced ability to induce HO-1, making them less able to deal with the damaging effects of haemolysis, and lead them to have a subsequently more serious form of the disease.

In summary, endothelial haem oxygenase is enhanced in response to blood from sickle patients, an effect which is further amplified if the patient has had a recent sickle crisis. The elevated endothelial haem oxygenase activity could act as a compensatory response to mitigate the acute effects of raised toxic haem levels and also confer long-term protection against further sickle crises developing.

Chapter 6. Haem oxygenase and its products regulate the interaction of sickle blood with the vascular endothelium

6.1 Introduction

SCD is characterized by recurring episodes of painful vaso-occlusion resulting in ischaemia/reperfusion injury and organ damage (Frenette and Atweh, 2007). The trigger for vaso-occlusive events is the adherence of sickle red blood cells to the vascular endothelium caused by abnormal expression of adhesion molecules. Another factor which may influence vaso-occlusion is vasoconstriction secondary to NO scavenging by haemoglobin which is prevalent in SCD (Reiter et al., 2002).

We have shown in the previous chapter that endothelial haem oxygenase is induced in the presence of sickle blood; we also observed increased haem oxygenase activity associated with exposure of cells to blood taken from sickle patients who had a recent crisis. We hypothesized that this may be a mechanism to protect against further vaso-occlusive events. Therefore, in the present study, we decided to investigate whether haem oxygenase or haem oxygenase breakdown products can modulate the adhesion of red blood cell from sickle blood to the endothelium. Furthermore, we explored the vasodilatory effect of CO on vessel relaxation after exposure to sickle blood. These investigations may help to clarify the role of haem oxygenase in the prevention or resolution of vaso-occlusive crises in SCD.

6.2 Objectives

- To explore the role of haem oxygenase in the regulation of sickle red blood cell adhesion to endothelial cells

- To investigate the specific effect CO and BV on the adhesiveness of sickle red blood cell to endothelial cells

- To examine how whole sickle blood, sickle red blood cells or plasma affect the ability of CO-releasing molecules (CO-RMs) to elicit vessel relaxation in an isolated aortic ring model

- To study the ability of NO donors to elicit vessel relaxation in isolated aortic rings exposed to sickle blood

6.3 Experimental protocol

Before the start of each experiment, stocks of all reagents were freshly prepared. Stock solutions of haemin and BV (1 mM) were prepared by dissolving the compounds in 0.1 M NaOH and then adding 0.01 M phosphate buffer at pH 7.4. CORM-3, CORM-A1, DEA-NO and PAPA-NO stocks were prepared using distilled water. iCORM-3 and iCORM-A1 were prepared as described in Materials and Methods. Whole blood was collected as described in the previous chapter.

6.3.1 Determination of red blood cell adhesion to endothelium

An *in vitro* static assay (Gravity sedimentation method) was used as a model for red blood cell-endothelial cell interaction. As we planned to investigate not only the interaction of red blood cells with endothelial cells, but also the influence of other components present in the blood, we chose initially to incubate endothelial cells with whole blood solution prepared in culture medium. Preliminary experiments were designed to determine the optimal percentage of blood solution needed for incubation and the length of exposure to blood required for quantifying the extent of red blood cell-endothelial cell interaction. Both normal and sickle whole blood solutions were prepared in culture medium (1, 5, 10, 20% solution) and incubated with endothelial cells for different periods of time (15, 30 and 45 min). The results indicated good reproducibility when endothelial cells were incubated with 5% whole blood solution for a period of 45 min. To investigate the effect of haem oxygenase induction or products of haem breakdown by haem oxygenase on red blood cell adhesion, endothelial cells were cultured in 6 well dishes and pre-incubated for 6 h with haemin (1, 10, 30 μM), or 30 min with the following: BV (0.1, 0.5, 1 μM); CORM-3 (1, 10, 30 μM);

CORM-A1 (1, 5, 10 μM). The negative controls iCORM-3 (30 μM) and iCORM-A1 (10 μM) were also incubated for 30 min prior to exposure of cells to blood (details of iCORM preparation are provided in section 2.3.3). The pre-treatments were followed by addition of 5% whole blood for 45 min to allow the adhesion process to occur. At the end of the incubation, non-adherent cells were removed by washing 3 times with Hanks balanced salt solution and adherent cells were counted using a light microscope. We also examined whether the effect of haem breakdown products on cell adhesion were due to direct interaction with the endothelium or with red blood cells. For this, cells were pre-incubated with BV (0.1, 0.5, 1 μM), CORM-3 (1, 10, 30 μM), CORM-A1 (1, 5, 10 μM) and iCORMs (30 μM iCORM-3 or 10 μM iCORM-A1) with the whole blood solution for 30 min, prior to addition to cells for the adhesion assay. In additional experiments, endothelial cells were pre-incubated with the haem oxygenase inhibitor SnPPIX (2, 20, 60 μM), in the presence of haemin (1, 10, 30 μM) for 6 h, to explore if haem oxygenase was responsible for the observed effect. SnPPIX is a synthetic haem analogue which has been demonstrated to act as a competitive inhibitor of haem oxygenase activity in various cell types (Kappas and Drummond, 1986). Therefore the concentration of SnPPIX used to treat endothelial cells was double the concentration of haem used, i.e. 1, 10 or 30 μM haem was incubated with 2, 20 or 60 μM SnPPIX, respectively; thus SnPPIX would compete with haemin for binding to haem oxygenase and diminish the activation of the enzyme. To explore whether the increased susceptibility to haemolysis characteristic of sickle blood was responsible for its higher adhesiveness compared to normal blood, experiments were devised to mimic haemolysis in normal blood. One millilitre of normal blood was lysed

using 4 ml hypotonic water lysis (Bryan et al., 2004). We assumed that all the red blood cells were lysed, therefore, a portion of the lysed blood was added to 5% whole normal blood to reach final free haemoglobin concentrations of 5, 10 or 15 μM. Cells were then exposed to these solutions for the adhesion assay. Finally, to investigate whether the mutation leading to HbS formation influences cell adhesion, cells were incubated with 5% whole normal blood solution to which HbS was added at concentrations of 5, 10 or 15 μM.

6.3.2 Experiments using the isolated aortic ring model

To investigate the effect of sickle blood on vessel relaxation, aortic rings were exposed to sickle blood and the relaxation response to the vasodilators CO and NO was measured. In brief, the aorta was excised from Sprague-Dawley rats and cut into aortic rings of approximately 2-3 mm. Rings were incubated in oxygenated buffer containing either 1% whole blood or 0.5% red blood cells or plasma solutions collected either from normal subjects or sickle patients. Control rings were exposed to buffer alone. After their initial pre-contraction to 1 μM phenylephrine, rings were challenged with 100 μM CO-RMs and the effect on relaxation measured. Two different CO-RM molecules were used: CORM-3 which rapidly liberates CO (half-life = 3.6 min) and CORM-A1 (half-life = 21 min) which has a slower CO release. In further experiments, aortic rings were incubated with 1% whole sickle or normal blood in the presence or absence of the NO donors: DEA-NO (100 μM), a fast-releaser of NO (half-life = 2 min) and PAPA-NO (100 μM), a slower releaser of NO (half-life = 16 min).

6.3.3 Statistics

Differences in the data among the groups were analyzed by using one-way ANOVA combined with the Bonferroni test for cell adhesion experiments and two-way ANOVA combined with Bonferroni test for aortic ring experiments. Values were expressed as means±SEM and differences between groups were considered to be significant at $P<0.05$.

6.4 Results

6.4.1 Optimisation of the assay for quantifying the adhesiveness of sickle red blood cell to endothelial cells

Cell adhesion assays have been widely used as models for exploring interactions between endothelial cells and red blood cells. Our preliminary experiments were devised to make efficient use of blood samples from sickle patients whilst still generating reproducible results. Endothelial cells were incubated for various periods of time (15, 30, 45 min) with sickle or normal blood solutions (1, 5, 10, 20%) prepared in culture medium. Our results showed that red blood cells from whole sickle blood adhered extensively to endothelial cells, as demonstrated in Figure 6-1 A. In contrast, incubation of whole normal blood solutions at any concentration did not result in adherence of red blood cells to the endothelial layer (Figure 6-1 B). This effect was observed at all the time points examined. The adhesion of sickle blood depended both on the concentration of the blood solution and time of exposure (Figure 6-1 A). Based on the results of our preliminary experiments and taking into account the limited availability of sickle blood, incubation of endothelial cells with 5% whole blood solution for 45 min was considered the optimal experimental approach to quantitatively measure cell adhesion using small quantities of sickle blood.

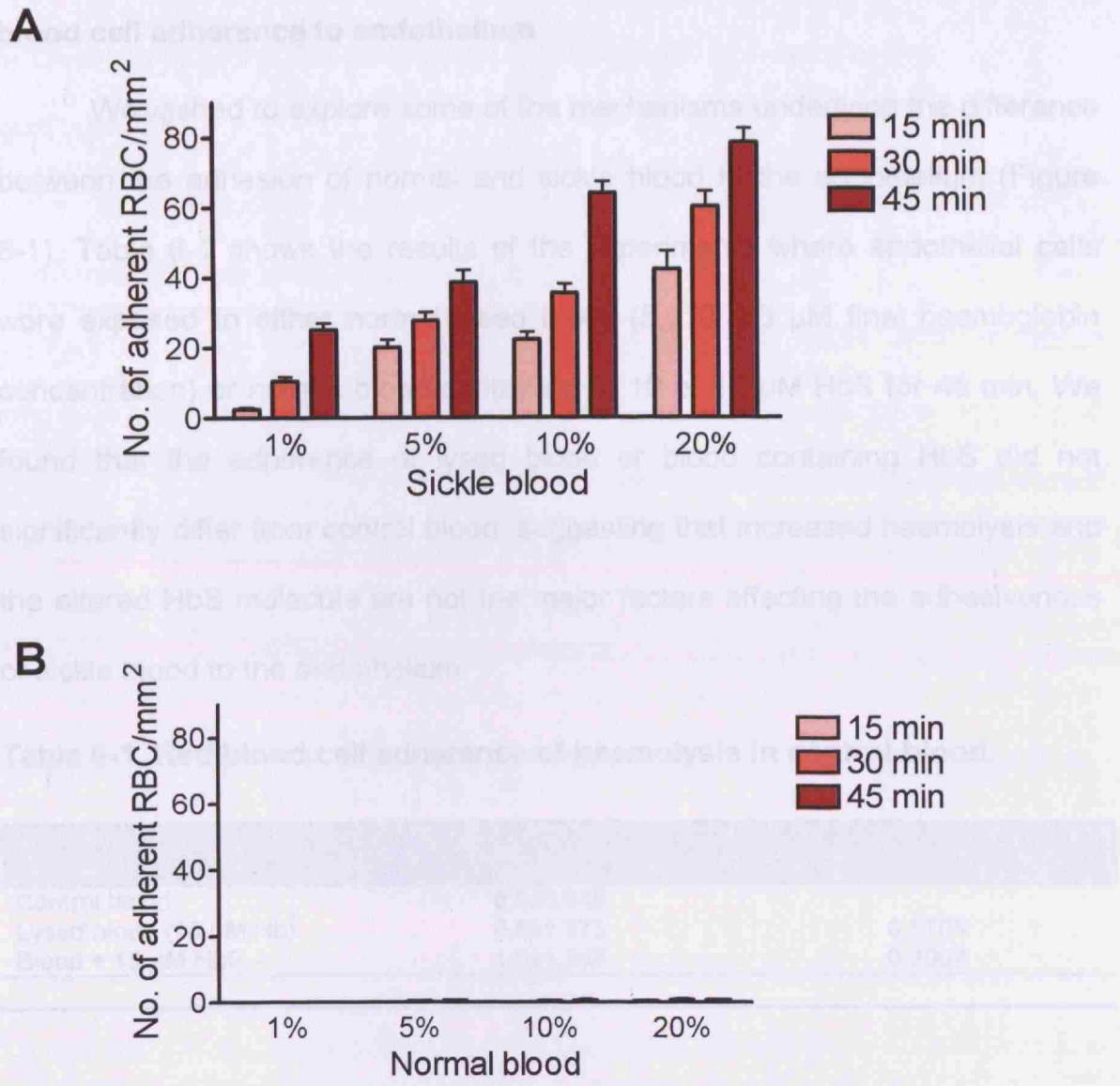


Figure 6-1. Adherence of red blood cells to the endothelium using normal or sickle blood

Endothelial cells were incubated for 15, 30 or 45 min with solutions (1, 5, 10, 20%) of whole sickle blood (A) or normal blood (B). Red blood cell (RBC) adhesion measurements were performed as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group.

6.4.2 Haemolysis of blood from normal subjects does not induce red blood cell adherence to endothelium

We wished to explore some of the mechanisms underlying the difference between the adhesion of normal and sickle blood to the endothelium (Figure 6-1). Table 6-1 shows the results of the experiments where endothelial cells were exposed to either normal lysed blood (5, 10, 15 μM final haemoglobin concentration) or normal blood containing 5, 10 or 15 μM HbS for 45 min. We found that the adherence of lysed blood or blood containing HbS did not significantly differ from control blood, suggesting that increased haemolysis and the altered HbS molecule are not the major factors affecting the adhesiveness of sickle blood to the endothelium.

Table 6-1. Red blood cell adherence of haemolysis in control blood.

	No of adherent cells/mm^2, mean±SD	P value vs. Control blood
Control blood	0.5±0.849	
Lysed blood (15 μM Hb)	0.6±1.075	0.8705
Blood + 15 μM HbS	1.0±1.247	0.3002

6.4.3 Induction of haem oxygenase and haem oxygenase-derived products modulate the adhesion of sickle red blood cells to the endothelium

Since induction of haem oxygenase in endothelial cells modulates the expression of adhesion molecules (Soares et al., 2004), it was of interest in our study to explore the effect of haem oxygenase and its products on the adhesion of sickle red blood cells. Figure 6-2 A shows that pre-treatment of endothelial cells with haemin (1, 10 and 30 μM) for 6 h caused a concentration-dependent reduction in sickle cell adhesion, with 30 μM haemin almost completely inhibiting the binding of sickle red blood cells to the endothelium. Co-incubation of endothelial cells with haemin and the haem oxygenase inhibitor SnPPIX re-established most of the adherence of sickle red blood cells (Figure 6-2 B), indicating that haem oxygenase may indeed have a role in the inhibition of endothelial red blood cell adhesion.

We wanted to further investigate the specific contribution of the haem oxygenase products BV and CO on red blood cell adherence and reasoned that BV or CO could interfere with the adhesion process by acting potentially on either endothelial cells or red blood cells. Interestingly, we found that pre-treatment of endothelial cells pre-treated with BV (0.1, 0.5, 1 μM) for 30 min prior to addition of sickle blood resulted in a concentration-dependent decrease in sickle cell adhesion (Figure 6-3 A), with 1 μM BV being the most effective concentration and reducing adhesion by approximately 50%. Notably, when red blood cells were incubated with BV (0.1, 0.5, 1 μM), we observed an almost complete abolition of red cell adhesion at all concentrations used (Figure 6-3 B).

To examine the effect of CO on red blood cell adhesion we employed two CO-RMs recently developed in our laboratory. The different chemical characteristics of CORM-3 and CORM-A1 affect their kinetic of CO release and consequently their potential effect on cells and tissues, as reviewed previously (Motterlini et al., 2005a). Pre-treating endothelial cells with CORM-3 or CORM-A1 elicited a concentration-dependent reduction in sickle cell adhesion (Figure 6-4 A and Figure 6-5 A). Importantly, neither iCORM-3 nor iCORM-A1 affected the adhesion response of sickle blood to endothelial cells. However, pre-incubation of sickle blood with either CORM-3 or CORM-A1 before addition to endothelial cells produced a different picture. Specifically, treatment of sickle blood with CORM-3 only marginally (and non-significantly) reduced sickle cell binding to endothelial cells (Figure 6-4 B), whereas pre-incubation with CORM-A1 exerted a substantial decrease in red blood cell adhesion (Figure 6-5 B). Interestingly, CORM-A1 had a greater effect on cell adhesion when added directly to sickle blood rather than to endothelial cells. We also noted that adhesion was lower when sickle blood was pre-treated with 10 μM iCORM-A1, although treatment of blood with 10 μM CORM-A1 inhibited adhesion to a much higher extent than iCORM-A1 (70% more). The fact that iCORM-A1 reduced cell adhesion may be a result of residual CO still bound to the CORM molecule, due to inefficient purging of CO molecules during the preparation of the inactive form (see Materials and Methods).

Collectively, these results indicate that prevention of sickle red cell adhesion is achieved not only via the induction of HO-1 in the endothelium, but also by treating either endothelial cells or sickle cell blood with haem oxygenase-derived products.

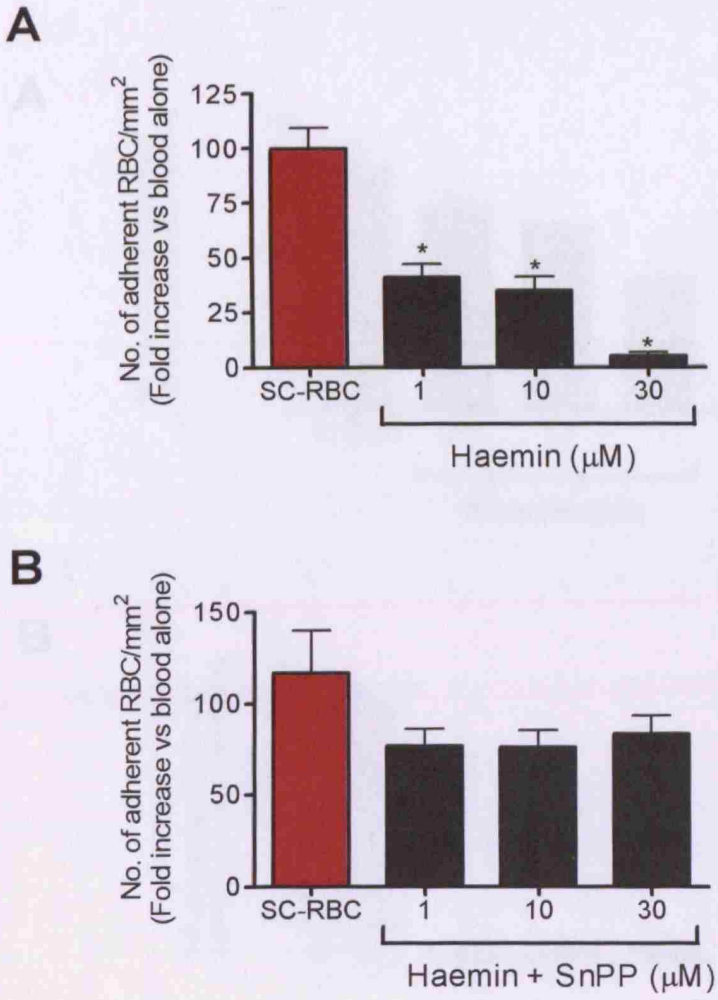


Figure 6-2. Pre-incubation with haemin decreases the adherence of sickle red blood cells (SC-RBC) to the endothelium

Endothelial cells were pre-incubated for 6 h with haemin (1, 10, 30 μM) (A) or haemin in the presence of the haem oxygenase inhibitor SnPPIX (2, 10, 60 μM) (B). Endothelial cells were then exposed to a 5% whole sickle blood solution for 45 min and red blood cell (RBC) adhesion measurements were performed as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. SC-RBC.

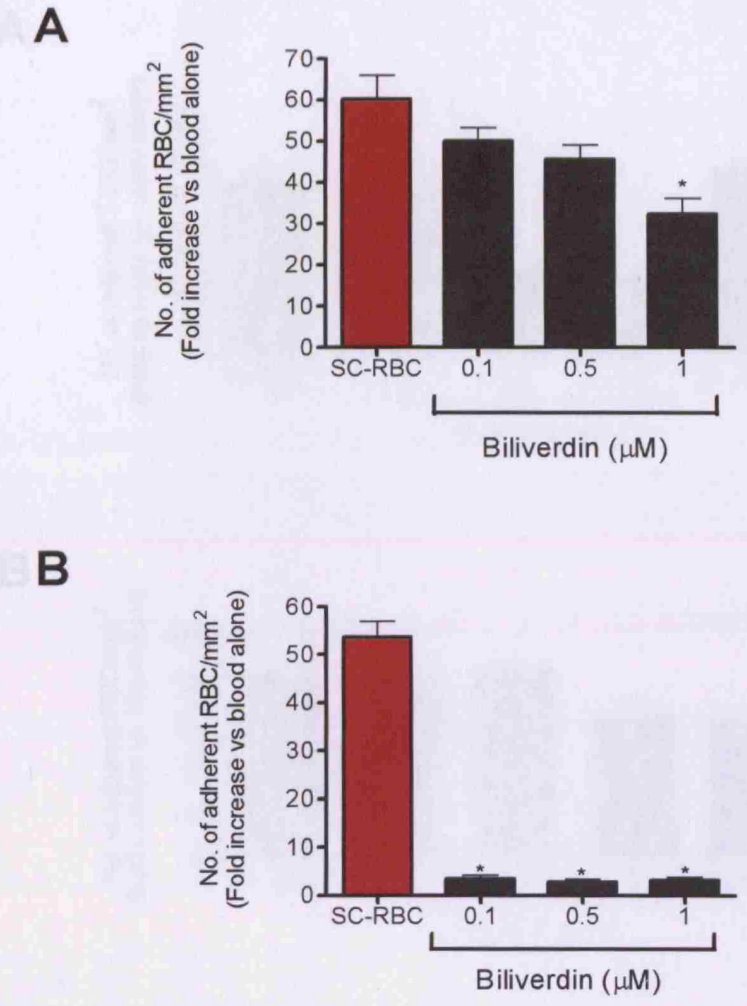


Figure 6-3. Pre-incubation with biliverdin reduces the adherence of sickle red blood cells (SC-RBC) to the endothelium

(A) Endothelial cells were pre-incubated for 30 min with biliverdin (0.1, 0.5, 1 μM) followed by exposure to 5% sickle blood solution for 45 min. (B) A 5% sickle blood solution was pre-incubated with biliverdin (0.1, 0.5, 1 μM) for 30 min followed by incubation with endothelial cells for 45 min. Red blood cell (RBC) adhesion measurements were performed as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. SC-RBC.

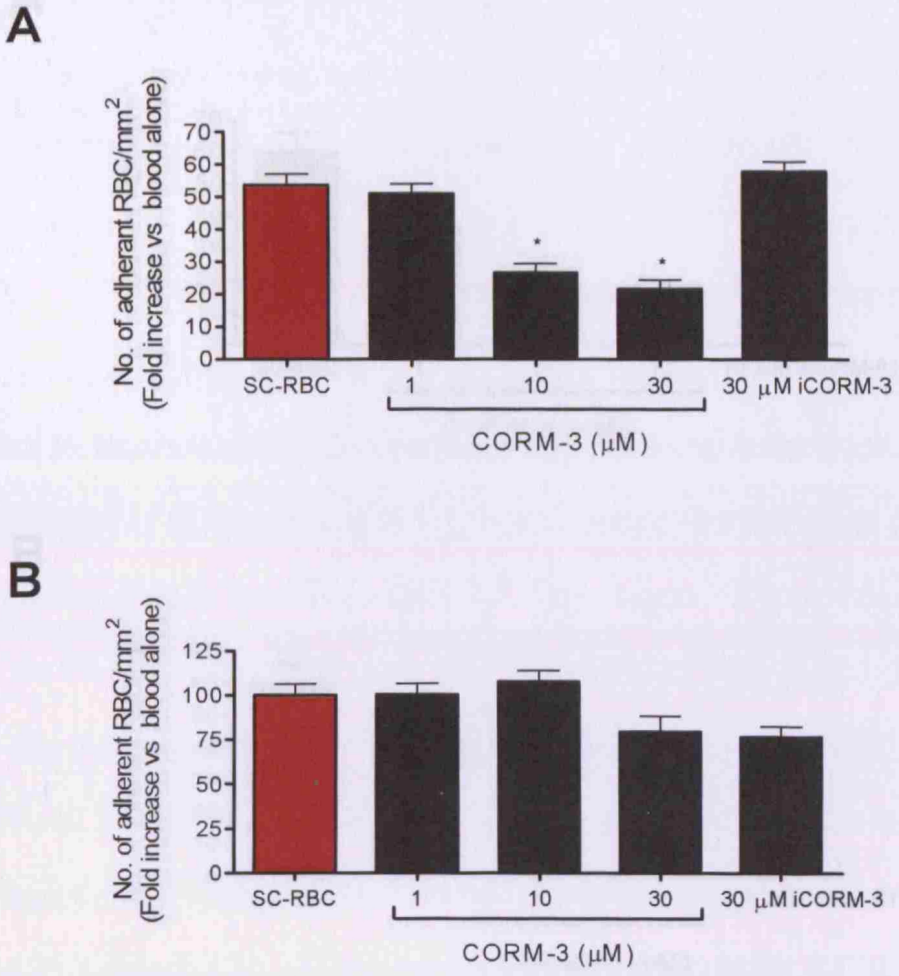


Figure 6-4. Pre-treatment with CORM-3 modulates adherence of sickle red blood cells to the endothelium

(A) Endothelial cells were pre-incubated for 30 min with CORM-3 (1, 10, 30 μM) or 30 μM iCORM-3 followed by exposure to 5% sickle blood solution for 45 min. (B) A 5% sickle blood solution was pre-incubated with CORM-3 (1, 10, 30 μM) or 30 μM iCORM-3 for 30 min followed by incubation with endothelial cells for 45 min. Red blood cell (RBC) adhesion measurements were performed as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. SC-RBC.

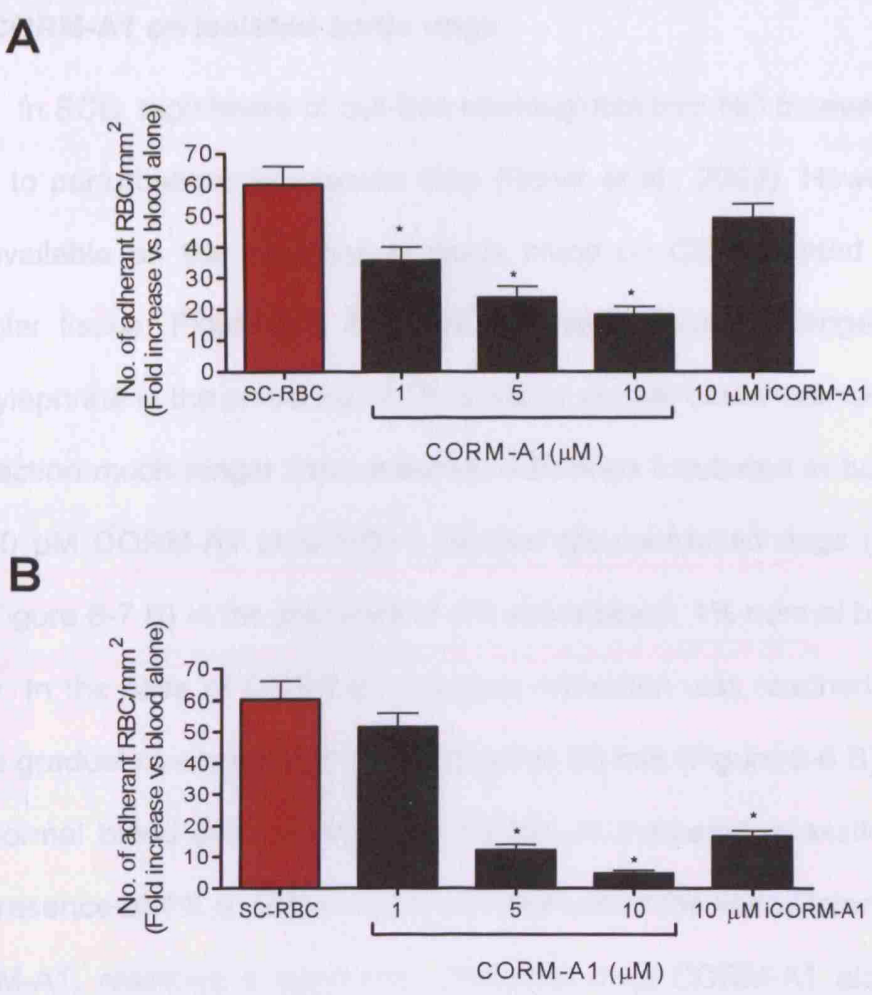


Figure 6-5. Pre-treatment with CORM-A1 reduces adherence of sickle red blood cells (SC-RBC) to the endothelium

(A) Endothelial cells were pre-incubated for 30 min with CORM-A1 (1, 5, 10 μM) or 10 μM iCORM-A1 followed by exposure to 5% sickle blood solution for 45 min. (B) A 5% sickle blood solution was pre-incubated with CORM-A1 (1, 5, 10 μM) or 10 μM iCORM-A1 for 30 min followed by incubation with endothelial cells for 45 min. Red blood cell (RBC) adhesion measurements were performed as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. SC-RBC.

6.4.4 Sickle whole blood modulates the vasodilatory effect of CORM-3 and CORM-A1 on isolated aortic rings

In SCD, high levels of cell-free haemoglobin limit NO bioavailability which leads to perturbations in vascular tone (Reiter et al., 2002). However, no data are available on the influence of sickle blood on CO-mediated relaxation of vascular tissue. Figure 6-6 A shows that aortic rings challenged with 1 μM phenylephrine in the presence of 1% sickle or normal blood maintained maximal contraction much longer than observed with rings incubated in buffer. Addition of 100 μM CORM-A1 or CORM-3 relaxed pre-contracted rings (Figure 6-6 B and Figure 6-7 B) in the presence of 1% sickle blood, 1% normal blood or buffer alone. In the case of CORM-A1 maximal relaxation was reached after 30 min and a gradual re-contraction occurred within 60 min (Figure 6-6 B). Addition of 1% normal blood did not affect the CORM-A1-mediated relaxation. However, the presence of 1% sickle blood slightly attenuated the vasodilatory response of CORM-A1, reaching a significant difference from CORM-A1 alone or in the presence of normal blood at 20 min time point (Figure 6-6 C). CORM-3 alone elicited a lower aortic relaxation compared to CORM-A1 (Figure 6-7) but was still maintained at 60 min. The presence of 1% normal or sickle blood exerted an attenuation of the vasodilatory response of CORM-3, but the difference was not statistically significant (Figure 6-7 A). In addition, there was no significant difference observed between the effect of normal and sickle blood on CORM-3 elicited relaxation (Figure 6-7 C). Therefore, the magnitude and length of relaxation differed between the two CO-RMs, with CORM-A1 eliciting a dramatically stronger relaxation response than CORM-3, both in the presence and absence of 1% blood.

In summary, the vasodilatory actions of CORM-3 and CORM-A1 are attenuated by the presence of sickle or normal blood. In addition, experiments with CORM-A1 demonstrate that blood from sickle patients has a significantly greater effect on inhibiting CORM-A1-mediated vasorelaxation than normal blood.

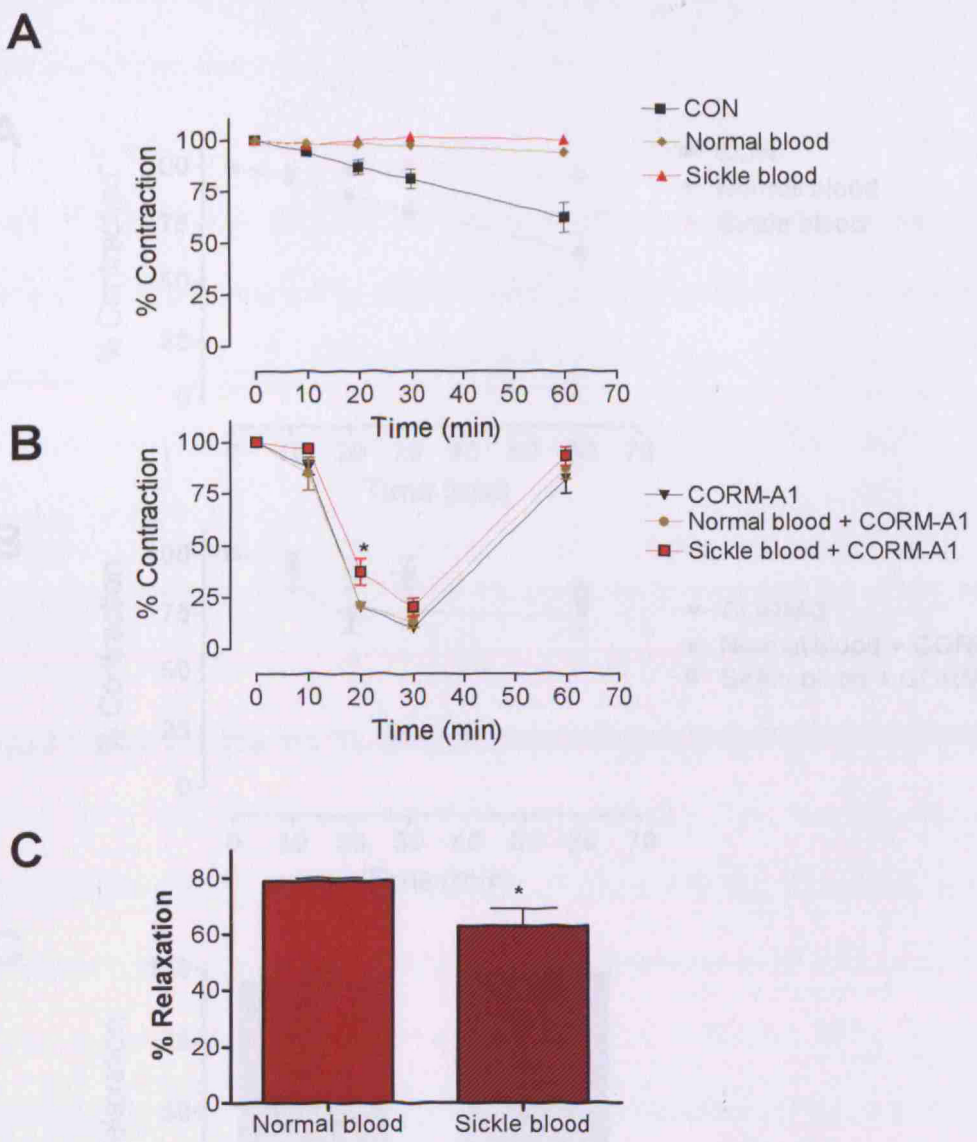


Figure 6-6. Sickle blood modulates vessel relaxation in response to CORM-A1

(A) Aortic rings suspended in Krebs buffer alone (CON), 1% normal (Normal blood) or sickle (Sickle blood) were challenged with 1 μM phenylephrine and contraction was followed over time. (B) At the peak of phenylephrine contraction, 100 μM CORM-A1 was added to rings incubated with Krebs buffer (CORM-A1), 1% normal blood (Normal blood + CORM-A1) or 1% sickle blood (Sickle blood + CORM-A1). (C) Vessel relaxation measured 20 min after addition of 100 μM CORM-A1 to aortic rings exposed to normal or sickle blood. Isolated aortic ring experiments were performed as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. Normal blood.

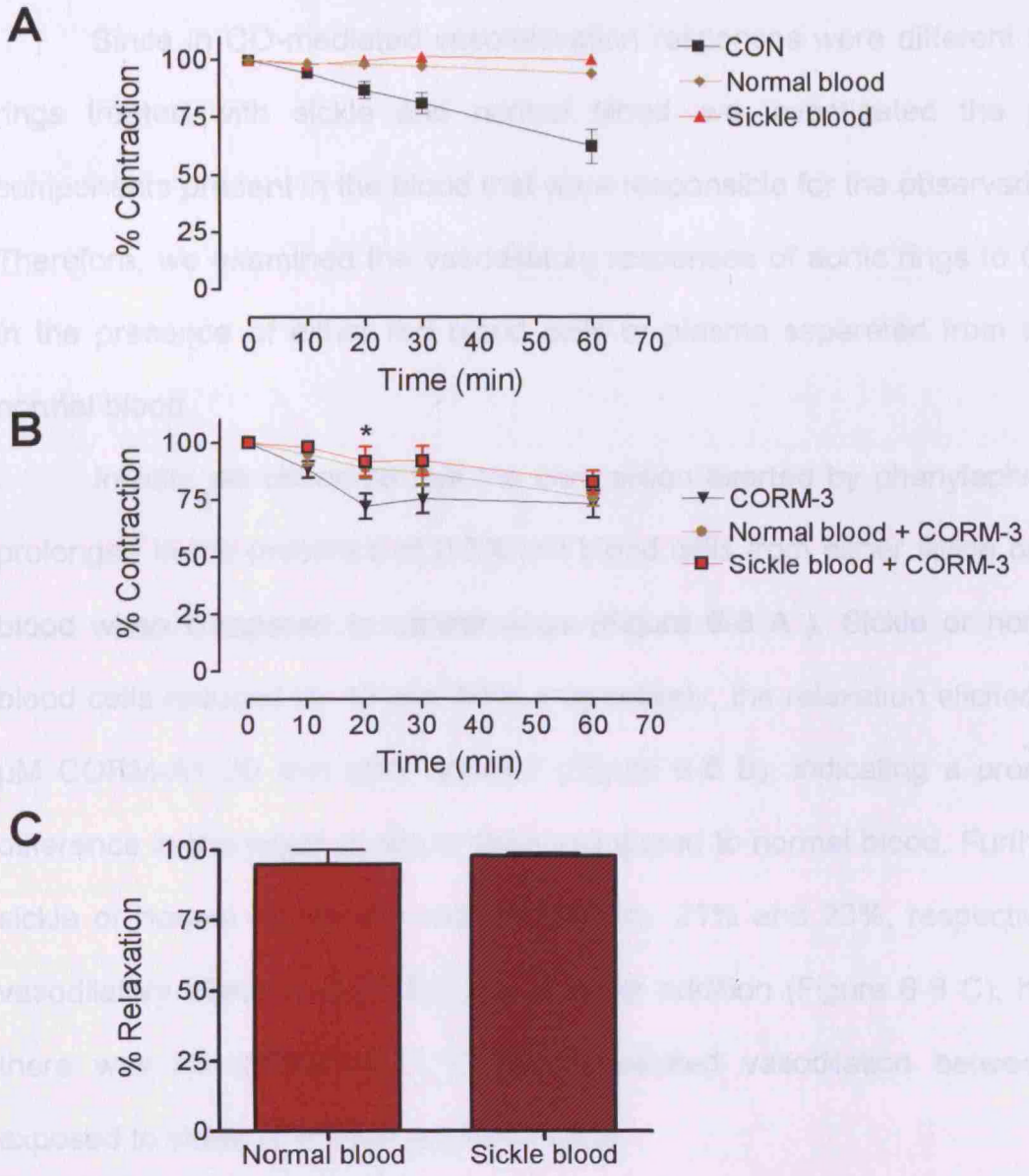


Figure 6-7. Sickle and normal blood modulates vessel relaxation in response to CORM-3

(A) Aortic rings suspended in Krebs buffer alone (CON), 1% normal (Normal blood) or sickle (Sickle blood) blood were contracted with 1 μM phenylephrine and contraction was followed over time. (B) At the peak of phenylephrine contraction, 100 μM CORM-3 was added to rings incubated with Krebs buffer (CORM-3), 1% normal blood (Normal blood + CORM-3) or 1% sickle blood (Sickle blood + CORM-3). (C) Vessel relaxation measured 10 min after addition of 100 μM CORM-3 to aortic rings exposed to normal or sickle blood. Isolated aortic ring experiments were performed as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. Normal blood.

6.4.5 Sickle red blood cells modulate the vasodilatory effect of CORM-3 and CORM-A1 on isolated aortic rings

Since in CO-mediated vasorelaxation responses were different in aortic rings treated with sickle and normal blood, we investigated the possible components present in the blood that were responsible for the observed effects. Therefore, we examined the vasodilatory responses of aortic rings to CO-RMs in the presence of either red blood cells or plasma separated from sickle or normal blood.

Initially we observed that the contraction exerted by phenylephrine was prolonged in the presence of 0.5% red blood cells from either sickle or normal blood when compared to control rings (Figure 6-8 A). Sickle or normal red blood cells reduced by 45 and 10%, respectively, the relaxation elicited by 100 μM CORM-A1 30 min after addition (Figure 6-8 B), indicating a pronounced difference in the effect of sickle blood compared to normal blood. Furthermore, sickle or normal red blood cells inhibited by 21% and 23%, respectively, the vasodilatory effect of CORM-3 20 min after addition (Figure 6-8 C); however, there was no difference in CORM-3-mediated vasodilation between rings exposed to sickle or normal red blood cells.

Interesting observations were also made when the effect of 0.5% normal or sickle plasma on CO-RMs relaxation was examined (Figure 6-9 B, C). Specifically, the presence of sickle or normal plasma inhibited by 47% and 39%, respectively, CORM-A1-mediated relaxation measured at 30 min after addition; with no difference observed between sickle and normal plasma. Similar results were obtained in the case of CORM-3, since the presence of sickle or normal plasma elicited a 16% reduction in vasorelaxation 20 min after the addition of

the compound, and again no differences were observed between the effect of sickle or normal plasma. These data suggest that plasma from either sickle or normal blood is capable of scavenging CO and limiting its vasodilatory action.

In summary, red blood cells or plasma from sickle or normal blood inhibited the vasodilatory properties of both CO-RMs. A significant difference was found in the vasodilatory action of CORM-A1 in the presence of sickle or normal red blood cells, where sickle red blood cells significantly diminished the action of CORM-A1. This effect was not seen with CORM-3 in the presence of red blood cells, or either CO-RMs in the presence of plasma.

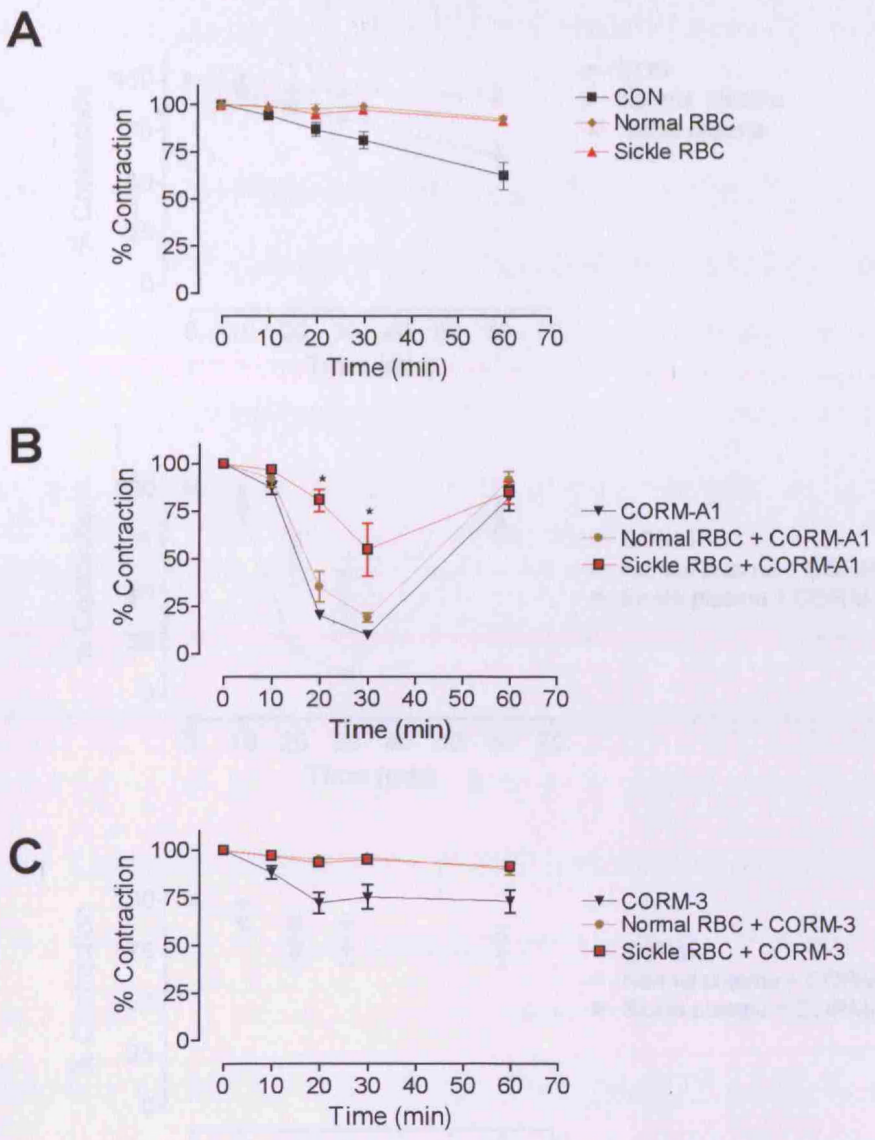


Figure 6-8. Sickle red blood cells modulate vessel relaxation in response to CO-RMs

(A) Aortic rings suspended in Krebs buffer alone (CON), 0.5% normal red blood cells (Normal RBC) or sickle red blood cells (Sickle RBC) were challenged with 1 μM phenylephrine and contraction was followed over time. (B) At the peak of phenylephrine contraction, 100 μM CORM-A1 was added to rings incubated with Krebs buffer (CORM-A1), 0.5% normal RBC (Normal RBC + CORM-A1) or 0.5% sickle RBC (Sickle RBC + CORM-A1). (C) At the peak of phenylephrine contraction, 100 μM CORM-3 was added to rings incubated with Krebs buffer (CORM-3), 0.5% normal RBC (Normal RBC + CORM-3) or 0.5% sickle RBC (Sickle RBC + CORM-3). Isolated aortic ring experiments were performed as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. Normal RBC.

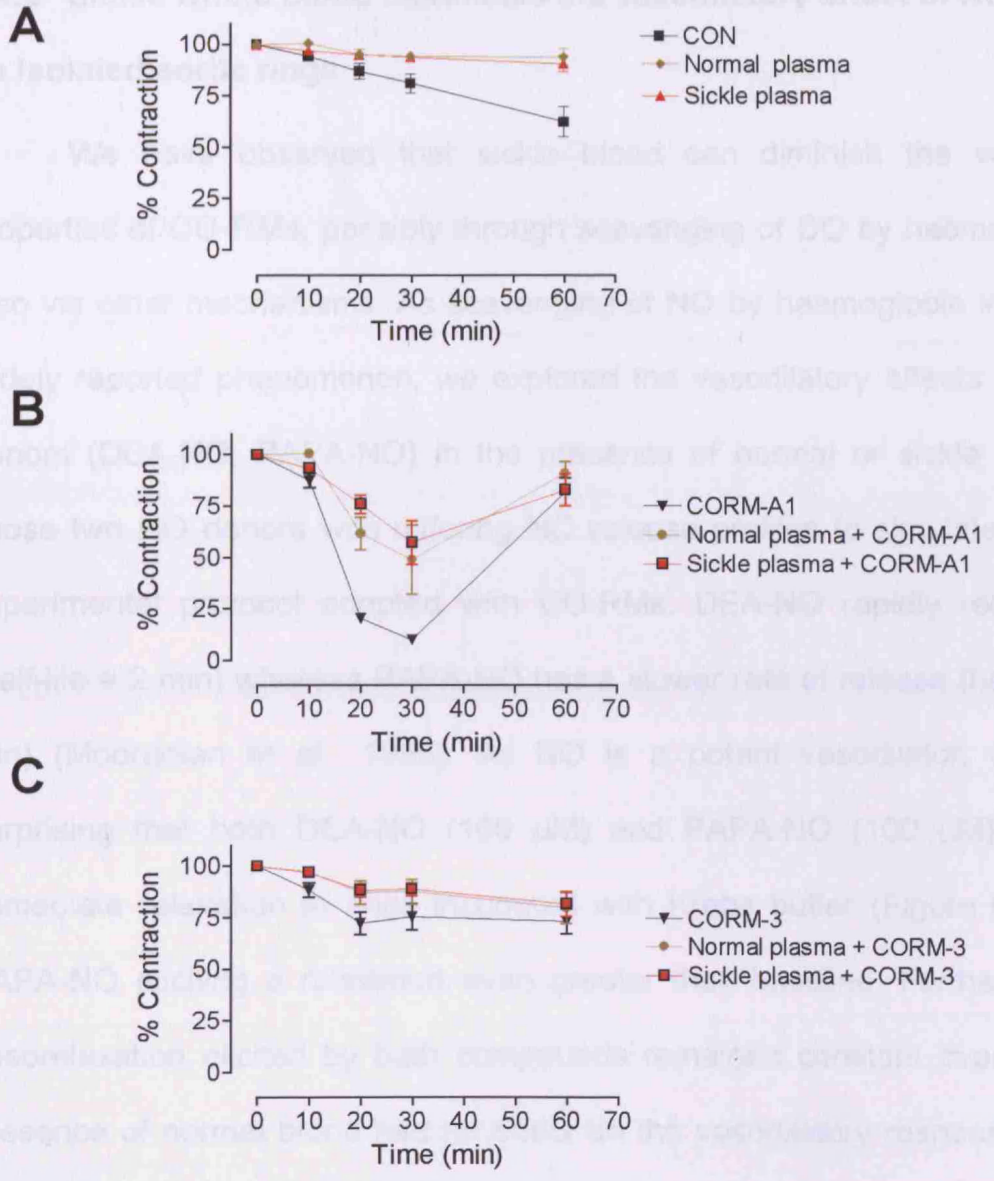


Figure 6-9. Sickle plasma modulate vessel relaxation in response to CO-RMs

(A)Aortic suspended in Krebs buffer alone (CON), 0.5% normal (Normal blood) or sickle (Sickle blood) plasma rings were challenged with 1 μM phenylephrine and contraction was followed over time. (B) At the peak of phenylephrine contraction, 100 μM CORM-A1 was added to rings incubated with Krebs buffer (CORM-A1), 0.5% normal plasma (Normal plasma + CORM-A1) or 0.5% sickle plasma (Sickle plasma + CORM-A1). (C) At the peak of phenylephrine contraction, 100 μM CORM-3 was added to rings incubated with Krebs buffer (CORM-3), 0.5% normal plasma (Normal plasma + CORM-3) or 0.5% sickle plasma (Sickle plasma + CORM-3. Isolated aortic ring experiments were performed as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. Normal plasma.

6.4.6 Sickle whole blood modulates the vasodilatory effect of NO donors on isolated aortic rings

We have observed that sickle blood can diminish the vasodilatory properties of CO-RMs, possibly through scavenging of CO by haemoglobin but also via other mechanisms. As scavenging of NO by haemoglobin in SCD is a widely reported phenomenon, we explored the vasodilatory effects of two NO donors (DEA-NO, PAPA-NO) in the presence of normal or sickle blood. We chose two NO donors with differing NO release profiles to simulate the same experimental protocol adopted with CO-RMs. DEA-NO rapidly releases NO (half-life = 2 min) whereas PAPA-NO has a slower rate of release (half-life = 16 min) (Mooradian et al., 1995). As NO is a potent vasodilator, it was not surprising that both DEA-NO (100 μM) and PAPA-NO (100 μM) produced immediate relaxation in rings incubated with Krebs buffer (Figure 6-10), with PAPA-NO eliciting a relaxation even greater than baseline. Furthermore, the vasorelaxation elicited by both compounds remained constant over 1 h. The presence of normal blood had no effect on the vasodilatory response of DEA-NO compared to control (Krebs buffer) (Figure 6-10 A). Surprisingly, the presence of sickle blood enhanced DEA-NO-mediated relaxation in comparison to normal blood or control and this effect was significant at 10 and 20 min after addition of DEA-NO.

The pattern of vasodilatory response elicited by PAPA-NO in the presence of blood was different from that observed with DEA-NO (Figure 6-10 B). The presence of both sickle and normal blood only slightly enhanced the

relaxation exerted by PAPA-NO and no difference was observed between the effect of sickle or normal blood.

In summary, NO elicited powerful vasorelaxation despite the presence of sickle or normal blood. In particular, sickle blood caused an enhancement of the vasodilatory properties of DEA-NO.

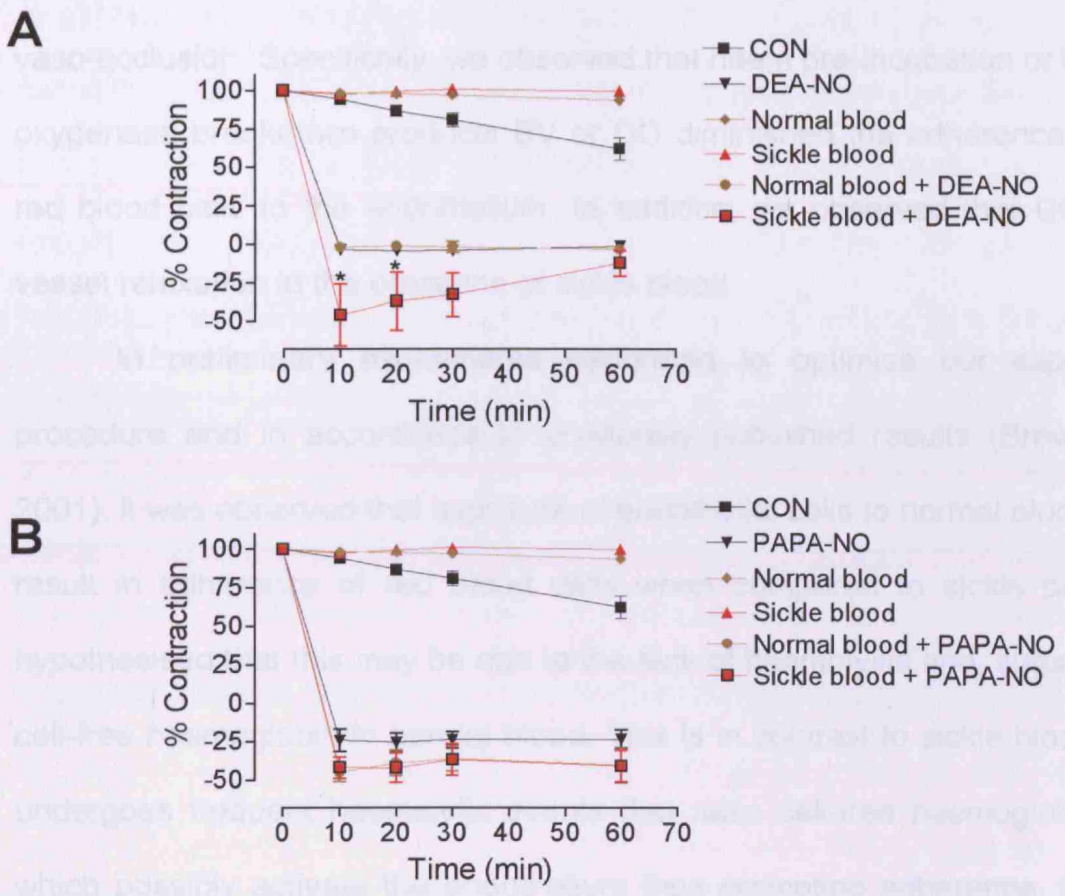


Figure 6-10. Sickle blood modulates vessel relaxation in response to NO donors

(A) Aortic rings suspended in Krebs buffer alone (CON), 1% normal (Normal blood) or sickle (Sickle blood) blood were challenged with 1 μM phenylephrine and contraction was followed over time. At the peak of phenylephrine contraction, 100 μM DEA-NO was added to rings incubated with Krebs buffer (DEA-NO), 1% normal blood (Normal blood + DEA-NO) or 1% sickle blood (Sickle blood + DEA-NO). (B) In further experiments, at the peak of phenylephrine contraction, 100 μM PAPA-NO was added to rings incubated with Krebs buffer (PAPA-NO), 1% normal blood (Normal blood + PAPA-NO) or 1% sickle blood (Sickle blood + PAPA-NO). Isolated aortic ring experiments were performed as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. Normal blood.

6.5 Discussion

In this chapter we have shown that haem oxygenase or haem oxygenase breakdown products can modulate factors associated with the development of vaso-occlusion. Specifically, we observed that haem pre-incubation or the haem oxygenase breakdown products BV or CO diminished the adherence of sickle red blood cells to the endothelium. In addition, we observed that CO elicited vessel relaxation in the presence of sickle blood.

In preliminary experiments performed to optimise our experimental procedure and in accordance to previously published results (Brown et al., 2001), it was observed that exposure of endothelial cells to normal blood did not result in adherence of red blood cells when compared to sickle blood. We hypothesised that this may be due to the lack of haemolysis and, subsequently, cell-free haemoglobin in normal blood. This is in contrast to sickle blood, which undergoes frequent haemolytic events that raise cell-free haemoglobin levels which possibly activate the endothelium thus promoting adherence. However, our experiments do not support this hypothesis since an increase in red blood cell adherence was not observed when normal blood was lysed or had added cell-free haemoglobin. These data suggest that there are other components in blood of sickle patients, different from haem or haemoglobin, that influence the adherence process. Indeed, chronic inflammation characteristic of SCD stimulates the production of a large number of circulating pro-inflammatory mediators which are not normally present in control subjects. These factors, together with oxidants produced in sickle blood (Frenette, 2002), could easily influence the activation of endothelial cells and subsequent adherence of red blood cells.

Pre-incubation of endothelial cells with haemin inhibited sickle red blood cell adhesion. We proposed that this effect was through the induction of haem oxygenase, a hypothesis that was confirmed by the observation that SnPPIX, a haem oxygenase inhibitor, diminished the effect of haemin on sickle red blood cell adhesion. However, SnPPIX did not elicit a complete reversal of the actions of haemin on cell adhesion. As SnPPIX is a competitive inhibitor of haem oxygenase activity, it is possible that some residual haem oxygenase activity remained and still modulated cell adhesion to a small extent. It is also important to mention that SnPPIX is a non-specific haem oxygenase inhibitor when used at high concentrations and will block the activity of all isoforms of the enzyme. Since endothelial cells express both HO-1 (inducible) and HO-2 (constitutive), the use of SnPPIX does not allow us to ascertain whether the increase in haem oxygenase activity was due to HO-1 induction. However there is vast literature showing that HO-2 is not inducible (apart from glucocorticoids) and HO-1 is up-regulated by haemin and oxidative stress (Maines, 1988), which are typical of SCD. Therefore based on previous sound evidence we assume that it is the HO-1 isoform of the enzyme that is being induced in our experiments. Nonetheless, further experiments involving specific HO-1 gene knockdown in endothelial cells would corroborate our findings. For example, small interfering RNA (siRNA) technology has been widely used to silence specific genes in mammalian cells via the introduction of synthetic double-stranded siRNA using plasmid or viral vectors (Leung and Whittaker, 2005). Such experiments would distinguish clearly between the contribution of endothelial HO-1 or HO-2 to our observed results.

Further experiments involving treatment with BV and also reduced the adherence of sickle red blood cells to the endothelium, which further confirmed that haem oxygenase was involved in this effect. These results could be explained by a reduction in the expression of endothelial adhesion molecules. Although haem itself has been shown to induce adhesion molecule expression in endothelial cells, its ability to induce HO-1 has been proposed to modulate this effect (Wagener et al., 1997). Indeed, Soares *et al* observed that up-regulation of HO-1 diminished the expression of adhesion molecules associated with endothelial cell activation (Soares et al., 2004). Furthermore, anti-inflammatory treatment in SCD inhibits endothelial cell activation and adhesion molecule expression and leads to a reduction in vaso-occlusion (Kaul et al., 2004). As haem oxygenase exerts potent anti-inflammatory actions, its induction in SCD could result in potential protective effects in the development of vaso-occlusive crisis. We also note that hydroxyurea treatment in SCD significantly lowers expression of adhesion molecules on sickle red blood cells (Gambero et al., 2007). As hydroxyurea has been shown to act as a NO donor (King, 2004) and NO activates haem oxygenase (Foresti et al., 1997; Motterlini et al., 1996a) we speculate that hydroxyurea can reduce red blood cell adhesion molecule expression via haem oxygenase.

Pre-incubation of endothelial cells with BV elicited a concentration-dependent reduction in red blood cell adhesion. However, pre-incubation of sickle blood with BV elicited an even more powerful reduction in red blood cell adhesion to endothelial cells. These findings may be explained by the potent anti-oxidant activity of BV (and BR) and indicate that treatment of sickle blood is a very effective strategy in decreasing adherence to the endothelium. BR has

been reported previously to have important protective anti-oxidant effects on sickle cell membranes (Dailly et al., 1998) and our results using BV suggest that the anti-oxidant action of BV reduces membrane damage and the consequent expression of adhesion molecules on red blood cells. Since HO-1-derived BR protects the endothelium by reducing endothelial activation and dysfunction (Kawamura et al., 2005) as well as limiting lipid peroxidation in sickle mice (Belcher et al., 2006), our data sustain the idea that BV can act on both endothelial and red blood cells to reduce cellular interaction.

We also observed that CO from CORM-3 and CORM-A1 inhibited the adhesion of sickle red blood cells to endothelium. Similarly to BV, the action of CO was more pronounced when red blood cells were incubated with CO-RMs, suggesting that sickle red blood cells play a dominant role in stimulating adhesion and that they represent a potential target for therapeutic intervention. HO-1-derived CO has been reported to have anti-inflammatory properties (Motterlini et al., 2003) and may help reduce endothelial cell activation and expression of adhesion molecules. Interestingly, CO gas administration inhibited vascular stasis (Belcher et al., 2003) in a transgenic mice model of SCD, thus potentially diminishing the risk of vaso-occlusive crisis. HO-1-derived CO also suppresses endothelial cell apoptosis (Brouard et al., 2000), and this finding might be relevant for SCD, as endothelial cell apoptosis sustains inflammation and promotes vascular thrombosis which may play a role in vaso-occlusion. In a study conducted more than 30 years ago, Beutler showed that CO limits haemolysis in SCD by increasing the life-span of red blood cells (Beutler, 1975); this action of CO could reduce activation of or damage to endothelial or red blood cells by hindering the release of pro-inflammatory

haemoglobin. In addition, inhalation of CO at low levels inhibits the expression of pro-inflammatory genes and promotes the production of anti-inflammatory cytokines (Belcher et al., 2003). These studies suggest a potential role for CO as a therapeutic agent in SCD. Collectively, we speculate that inhibition of red blood cell adhesion to the endothelium by CO and BV contributes to inhibition of vaso-occlusion in SCD.

CO is very similar to NO and can mimic some of its functions, such as interacting with signal transduction pathways and vessel relaxation. NO has an important role in the pathogenesis of SCD where a reduction in basal and stimulated NO production and bioavailability has been described (Reiter et al., 2002). Haemolysis induced elevated plasma haemoglobin and excessive ROS production both act to reduce NO bioavailability either through scavenging by haemoglobin or formation of reactive nitrogen species. One of the most important consequences of reduced NO availability is the effect on vessel tone resulting in vasoconstriction, which could contribute to subsequent vaso-occlusion. We suggest that HO-1-derived CO, relatively inert compared to NO due to its lack of unpaired free electrons, may induce vasodilation in SCD and compensate for the reduced bioavailability of NO.

We observed that CO liberated from CORM-A1 relaxed vessels exposed to sickle and normal blood, although sickle blood reduced CORM-A1-mediated relaxation compared to normal blood. The increased levels of free haemoglobin present in sickle blood could scavenge CO and reduce its biological effects. This hypothesis is corroborated by our experiments where aortic rings were exposed to red blood cells or plasma alone, showing that sickle red blood cells inhibited CORM-A1-mediated vasorelaxation to a higher degree than normal

red blood cells. However, the effect of sickle red blood cells was more pronounced than that observed with whole sickle blood, suggesting that the plasma component of whole blood counter-balances the effect of red blood cells. Mosseri and colleagues reported that sickle red blood cells inhibited vasorelaxation and attributed the effect to the presence of free HbS and interaction of abnormal red blood cell membranes with the vessel wall (Mosseri et al., 1993). The authors also observed that the effect could be reversed by addition of anti-oxidants. Whether haem oxygenase, through the production of BV/BR might ameliorate abnormal vessel tone in SCD remains to be established.

The differences in vasorelaxation responses observed when rings were exposed to normal or sickle red blood cells may also indicate differences in binding of CO to HbA and HbS. Although a highly speculative idea, HbA binding to CO may maintain its bioavailability, whereas HbS binding may limit the biological action of CO. Indeed, abnormal binding of NO to HbS containing red blood cells has been demonstrated in SCD (Pawloski et al., 2005) and there may be similar anomalous interactions with CO.

Further experiments exploring the vasodilatory effects of CORM-A1 on rings exposed to plasma showed inhibition of vasorelaxation in the presence of both sickle and normal plasma. However, no differences were observed between plasma separated from sickle or normal blood. These data suggest that plasma in general is capable of scavenging CO and limiting its actions. However, the presence of cell-free haemoglobin in plasma might cause this effect. It is possible that separation of red blood cells from plasma may have resulted in some accidental lysis of red blood cells, thus raising plasma levels of

haemoglobin. Although more free haemoglobin would be expected in sickle than normal blood, even the small quantities present in normal blood may be sufficient to decrease the actions of CO.

In contrast to CORM-A1, CORM-3 did not elicit strong vasorelaxation in rings incubated with whole blood, red blood cells or plasma from sickle patients or control subjects. In addition, the CORM-3-mediated vasodilatory response was similarly inhibited by sickle and normal blood. The different kinetics of CO release by CORM-3 and CORM-A1 may explain the difference in the effects exerted by the two compounds.

In comparative experiments with two NO donors, we observed that both DEA-NO and PAPA-NO produced powerful and immediate vasodilation in vessels. Unsurprisingly, NO elicited greater vasodilation than that obtained with CO-RMs. Interestingly, the presence of sickle blood enhanced the vasodilation produced by DEA-NO in comparison to normal blood or control. As more free haemoglobin is present in sickle blood, enhanced scavenging of NO would be expected, thus causing vasoconstriction. The possibility that free haemoglobin actually facilitates the release of NO from DEA-NO or that binding of haemoglobin to NO enhances its bioavailability may account for our results. In contrast, even though PAPA-NO elicited enhanced vasodilation in the presence of sickle or normal blood, there was no difference between the two blood groups. As in the case of CO-RMs, the effects of the two NO donors may be explained by the different kinetics of NO release of the two molecules. It has been reported that the ability of sickle red blood cells to scavenge NO is reduced (Olmos et al., 2002) and that sickle red blood cells exhibit an abnormal processing of intracellular Hb-NO (Gow et al., 1999), suggesting that the

peculiar behaviour of sickle red blood cells is responsible for some of our observed results.

Even though NO is a more potent vasodilator than CO, its presence is severely reduced in SCD (Hsu et al., 2007). It is possible that CO has a role in controlling vascular tone in sickle patients, in an attempt to compensate for abnormal vessel tone due to reduced plasma NO. In other words, the high levels of haemolysis in SCD could provide sufficient substrate for haem oxygenase to produce CO which could help counteract the reduced bioavailability of NO. Indeed, high levels of carboxyhaemoglobin have been found to correlate with haemolysis in SCD (Sears et al., 2001), indicating production of CO in this disease. Interestingly, a recent British study investigating the relationship between air quality and the number of hospital admissions caused by acute pain in sickle patients found a correlation between high CO levels and reduced admissions, as opposed to an increase in admissions when atmospheric NO and CO levels are low (Yallop et al., 2007). These results are intriguing and possibly indicate an important role for CO in the prevention of sickle crisis. Accordingly, the above study sustains our hypothesis that haem oxygenase-derived CO or exogenously administered CO could function as a therapeutic agent in SCD.

A possible role for haem oxygenase in the prevention or resolution of pathological processes which may lead to vaso-occlusion in SCD is postulated in Figure 6-11. In brief, induction of endothelial haem oxygenase could reduce the expression of adhesion molecules of both red blood cells and endothelium and prevent adhesive interactions through the action of its breakdown products CO and BV. These products could also protect red blood cell membranes and

limit red blood cell sickling. Production of CO could also counteract the vasoconstriction caused by NO scavenging, thus preventing vascular stasis that leads to sickling of red blood cells and consequent vaso-occlusion.

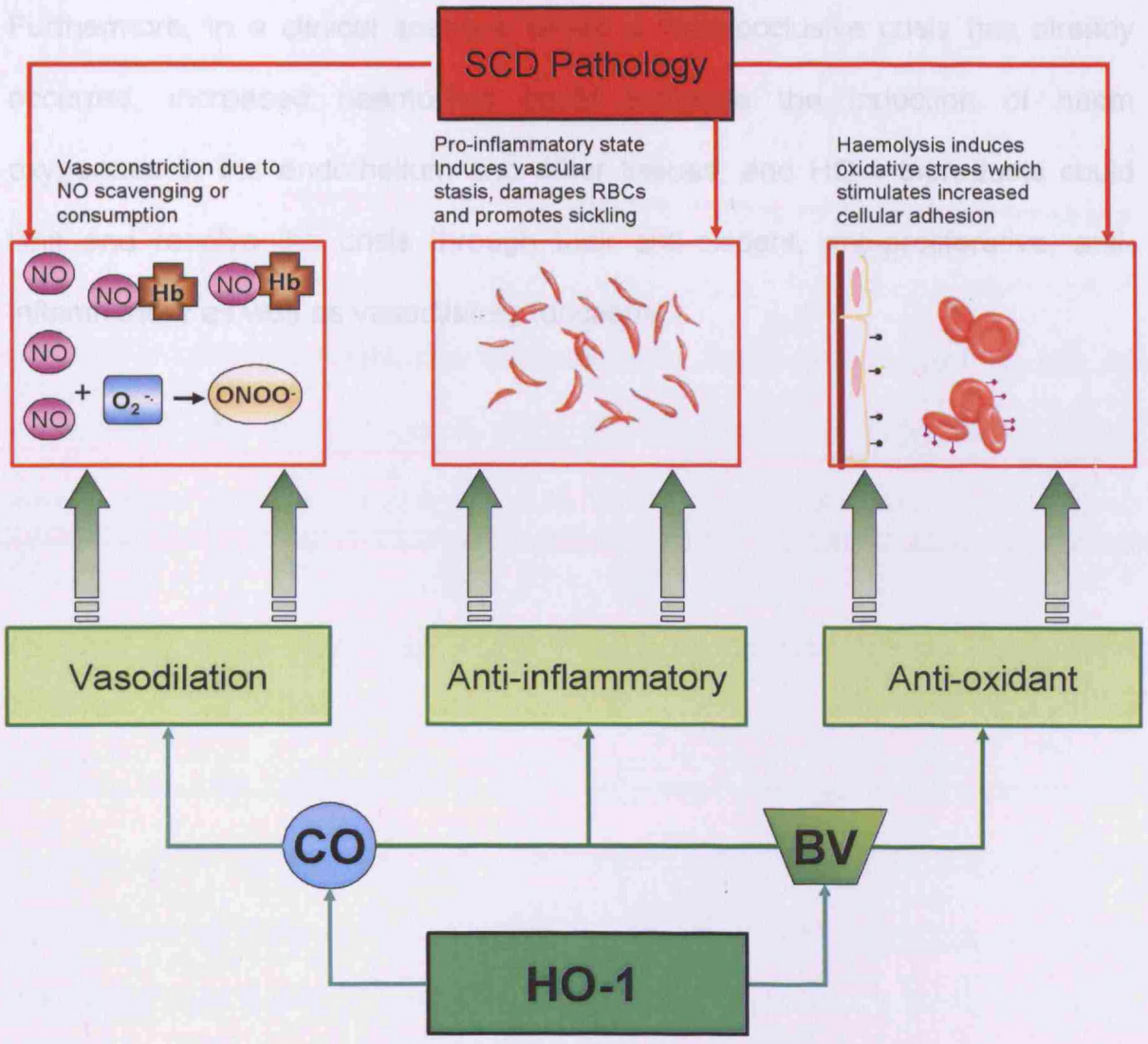


Figure 6-11. Proposed role for haem oxygenase in prevention of vaso-occlusion in SCD

Induction of haem oxygenase-1 (HO-1) in SCD results in the production of carbon monoxide (CO) and biliverdin (BV), which counteract the pathological processes present in the disease. The vasodilatory action of CO can mitigate the reduction in nitric oxide (NO) bioavailability caused by scavenging of haemoglobin (Hb) or consumption by superoxide ($O_2^{\cdot-}$) or peroxynitrite ($ONOO^-$). The anti-inflammatory and anti-oxidant properties of BV and CO can diminish the pro-inflammatory and pro-oxidant environment that results from red blood cell (RBC) sickling and haemolysis, thus reducing RBC damage and decreasing adhesion molecule expression in both RBCs or endothelium.

Furthermore, in a clinical scenario where a vaso-occlusive crisis has already occurred, increased haemolysis could stimulate the induction of haem oxygenase in the endothelium and other tissues, and HO-1 biproducts could limit and resolve the crisis through their anti-oxidant, anti-proliferative, anti-inflammatory as well as vasodilatory functions.

Chapter 7. Exploring the effect of HO-1 inducers on erythroid progenitor cells (K562)

7.1 Introduction

K562 are erythroid progenitor cells derived from the bone marrow of a patient with chronic myelogenous leukaemia (Lozzio and Lozzio, 1975). These cells are characteristically bi-potential, in other words, they can be stimulated to express erythroid or megakaryocytic properties depending on the stimulus. As such, K562 cells offer an excellent experimental model to study the molecular mechanisms of erythroid differentiation. For instance, following treatment with haemin or other inducing agents (like anthracyclines, sodium butyrate, and resveratrol) K562 cells can differentiate into haemoglobin-producing cells (Tsiftsoglou et al., 2003). These cells have been used as an invaluable tool in studying the regulation of foetal and embryonic haemoglobin expression, two haemoglobin subtypes that decrease the clinical severity of haemoglobinopathies such as SCD (Sauntharajah and DeSimone, 2004) and ß-thalasseamia (Lal and Vichinsky, 2004). In fact, it is the erythroid progenitor cells which provide a target for the action of hydroxyurea in SCD (Steinberg, 2002), where the drug stimulates the production of HbF, thus reducing the concentration of the mutant HbS in sickle red blood cells and diminishing the likelihood of sickling.

Exposure of endothelial cells to hydroxyurea induces haem oxygenase activity in the presence of haem. Haem oxygenase protects the endothelium from oxidative stress though its breakdown products (Immenschuh and Schroder, 2006), however, the potential role of haem oxygenase in K562 cells is

yet to be fully understood. It has been proposed that haem oxygenase may have a role as a directional switch between differentiation of K562 cells into erythroid or megakaryocytic cell lines (Koiso et al., 1999). Specifically, haem oxygenase induction in K562 cells is reported to induce differentiation to monocytes, whereas its inhibition results in erythroid differentiation. As hydroxyurea has been observed to induce endothelial haem oxygenase and the role of haem oxygenase in K562 needs to be fully explored, a preliminary set of experiments were designed to determine the response of K562 cells to hydroxyurea and common haem oxygenase inducers and to investigate whether haem oxygenase or its products affect cell viability. The consequent findings may help to better elucidate the therapeutic effect of hydroxyurea in SCD, with the potential involvement of haem oxygenase.

7.2 Objectives

- To explore the effect of various known haem oxygenase inducers on haem oxygenase activity levels in K562 cells

- To investigate whether co-incubation of haem and hydroxyurea can influence haem oxygenase activity levels in K562 cells

- To assess whether co-incubation of haem and hydroxyurea affects K562 cell viability

- To examine whether the haem oxygenase breakdown products CO and BV influence viability of K562 cells

7.3 Experimental protocol

The detailed methods used in the experiments described in the following section can be found in the Materials and Methods chapter. All stock solutions were freshly prepared. Hydroxyurea and thymidine (10 mM) were solubilised in distilled water. Haemin (1 mM) was dissolved in 0.1 M NaOH and 0.01 M phosphate buffer at pH 7.4. A stock solution of tin protoporphyrin IX (SnPPIX) was prepared by dissolving the compound in 0.1 M NaOH and was protected from light with aluminium foil. DETA-NO (10 mM) was solubilised in 0.01M NaOH. Curcumin was prepared in ethanol.

7.3.1 Incubation of cells with reagents

To investigate the effect of classic inducers of HO-1, K562 cells were exposed for 6 h to either haemin (15 μM, 30 μM, 50 μM), curcumin (10 μM, 20 μM, 50 μM), or the NO donor DETA-NO (50 μM, 100μM, 300μM) and collected for the haem oxygenase activity assay. The range of concentrations used was based on previous assessments of haem oxygenase activity in other cell types. K562 cells were also treated with medium alone as a control. In additional experiments, K562 cells were incubated with haemin (5 μM, 15 μM, 30 μM, 50 μM) for 20 h and haem oxygenase activity determined.

The effect of hydroxyurea (100, 300 and 500 μM) on haem oxygenase activity was also investigated by exposing K562 cells to the drug for 18, 24, 48 and 72 h. In another set of experiments, cells were incubated for 24 h with 50 μM, 100 μM and 200 μM thymidine (a pharmacological agent which stimulates haemoglobin synthesis as well reportedly inducing enzymes of haem degradation, including HO-1 in K562 cells (Trakshel et al., 1987)), and haem oxygenase activity measured. K562 cells were also incubated with 15 μM

haemin in the presence or absence of 300 μM hydroxyurea for 6 and 18 h followed by measurement of haem oxygenase activity.

The effect of haemin and hydroxyurea on cell viability was investigated. Firstly, cell damage was induced by challenging K562 cells with hydrogen peroxide (H_2O_2), a generator of oxidative stress, at 0.5 mM, 1 mM and 2 mM concentration for 24 h. Cells were also incubated with 1% Triton as a positive control. At the end of the incubation, LDH levels were measured in culture medium. In further experiments, K562 cells were pre-incubated with 15 μM haemin, 300 μM hydroxyurea or a combination of haemin and hydroxyurea for 18 h followed by addition 1 mM H_2O_2 for 24 h. Similar experiments were conducted in the presence of the haem oxygenase inhibitor SnPPIX (15 μM) to establish whether haem oxygenase protects H_2O_2-mediated damage (1 mM for 24 h).

To investigate the effect of haem oxygenase degradation products on H_2O_2-induced cytotoxicity, K562 cells were incubated with 1, 5 or 10 μM CORM-3 or CORM-A1 for 1 h, followed by exposure for 24 h with 1 mM H_2O_2. Cells were also incubated with the negative controls iCORM-3 and iCORM-A1 (10 μM). K562 cells were also pre-incubated for 1 h with 0.1, 0.5 or 1 μM BV prior to challenge with 1 mM H_2O_2 for 24 h.

7.3.2 Haem oxygenase activity assay

Haem oxygenase activity was determined in endothelial cells at different times after treatment as described in section 2.7 Briefly, harvested cells were subjected to three cycles of freeze-thawing before addition to a reaction mixture containing NADPH, rat liver cytosol as a source of BVR, and the substrate haemin. The reaction was conducted at 37 °C in the dark for 1 h, terminated by

the addition of 1 ml chloroform, and the extracted BR was calculated by the difference in absorbance between 464 and 530 nm (ε = 40 $mM^{-1}cm^{-1}$).

7.3.3 Lactate dehydrogenase assay

K562 cell viability was determined using the LDH assay. LDH is present in all cells as a stable cytoplasmic enzyme. Cell plasma membrane damage will result in LDH being rapidly released into cell culture supernatant. The LDH based cytotoxicity detection kit (Roche Diagnostics, UK) provides a colorimetric assay for the quantification of cell death and cell lysis dependent on the measurement of LDH activity released from the cytosol of damaged cells into the supernatant. The assay was carried out according to the manufacturers' instructions.

7.4 Results

7.4.1 Response of K562 cells to classic HO-1 inducers

Haem oxygenase is a protective protein which has been shown in many cell types to protect against the effects of oxidative damage. It can be induced by a number of agents and in studies already reported in this thesis its induction has been demonstrated in bovine endothelial cells, murine macrophages, porcine renal epithelial cells and human Girardi cardiomyocytes by haem and NO. The role of haem oxygenase in K562 cells is yet to be fully understood, and it would be interesting to investigate if classic inducers of haem oxygenase in other cell types (such as haemin, NO and others) would elicit the same response on haem oxygenase activity in K562 cells.

Contrary to our expectations, incubation of K562 cells for 6 h with haemin, curcumin or DETA-NO failed to augment haem oxygenase activity in K562 cells, actually producing a significant decrease in activity compared to control. Figure 7-1 A shows that haem caused an approximate 50% reduction in basal haem oxygenase activity at all concentrations used (15, 30 and 50 μM). Similarly, curcumin elicited a concentration-dependent reduction in haem oxygenase activity with 50 μM curcumin exhibiting an almost 50% reduction (Figure 7-1 B). The NO donor DETA-NO did not significantly change haem oxygenase activity at any of the concentrations employed (50, 100 and 300 μM) (Figure 7-1 C).

As haem is also the substrate for haem oxygenase activity, its effect on K562 cells was further explored using a wider concentration range for a longer

time interval. Figure 7-2 shows that incubation of cells with haemin (5, 15, 30 and 50 μM) for 20 h caused a concentration dependent inhibition of haem oxygenase activity, with 50 μM haem reducing the activity to approximately 20% of control.

In summary, our experiments indicated that the classic inducers of HO-1 (haem, curcumin and NO) failed to increase haem oxygenase activity in K562 cells. In fact, haem and curcumin reduced activity to levels below control, suggesting that the mechanism(s) responsible for induction of haem oxygenase in K562 cells may differ from other cell types.

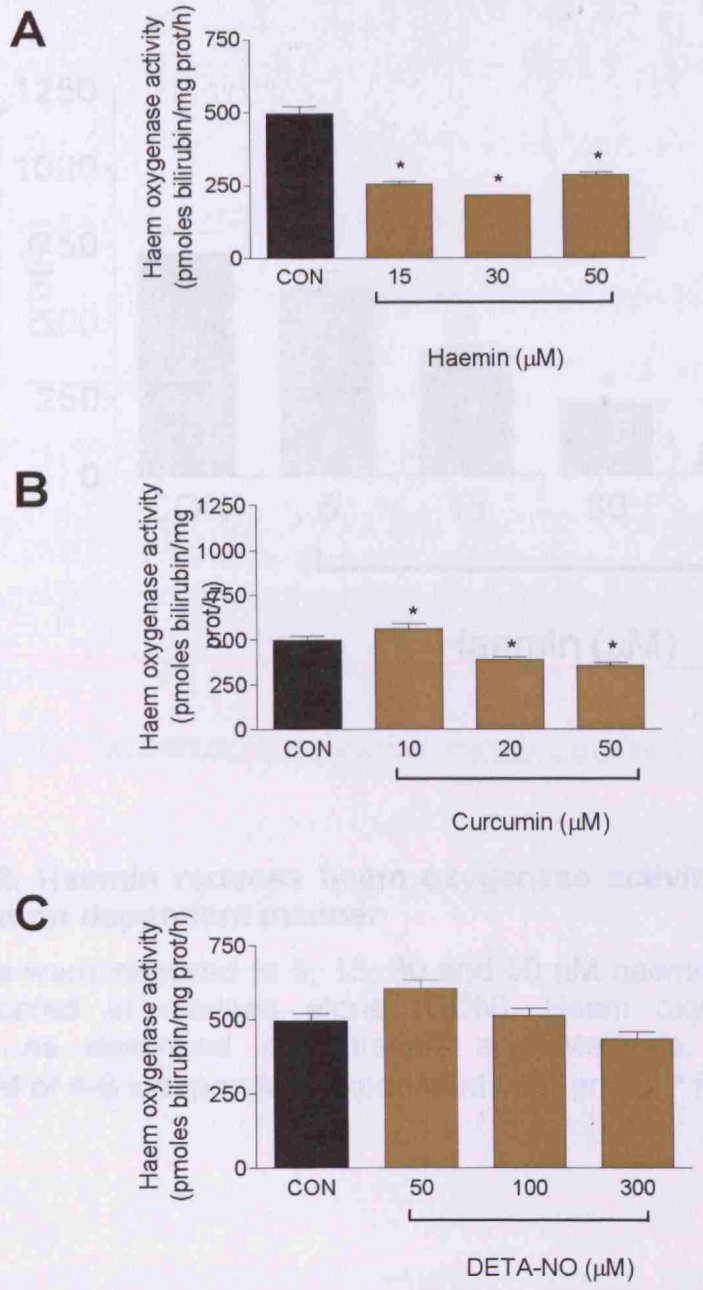


Figure 7-1. Classic HO-1 inducers do not increase haem oxygenase activity in K562 cells

K562 cells were treated for 6 h with: (A) 15, 30 and 50 μM haem; (B) 10, 20 and 50 μM curcumin; (C) 50, 100 and 300 μM DETA-NO. Cells were also incubated in medium alone (CON). Haem oxygenase activity was measured as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. CON.

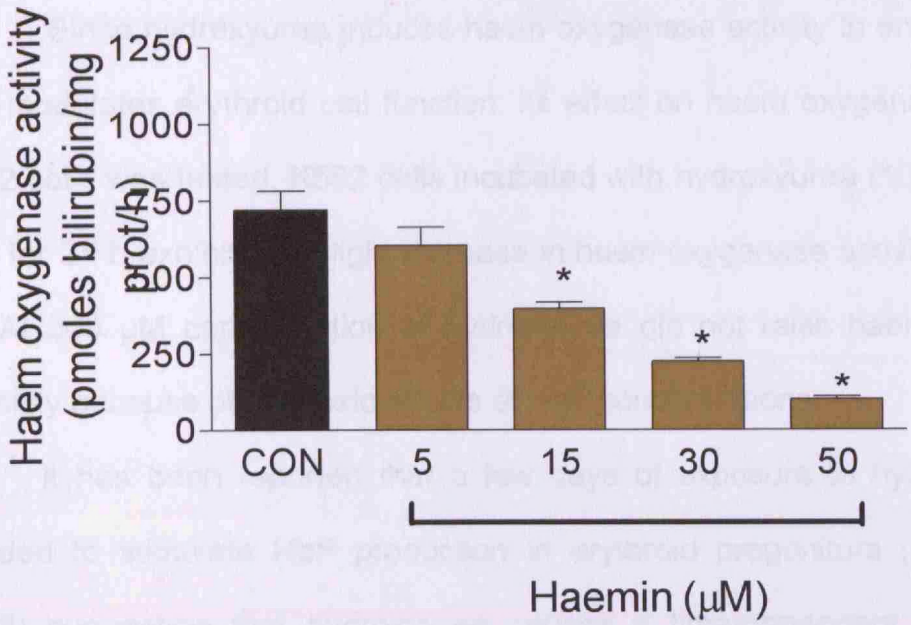


Figure 7-2. Haemin reduces haem oxygenase activity of K562 cells in a concentration-dependent manner

K562 cells were exposed to 5, 15, 30 and 50 μM haemin for 20 h. Cells were also incubated in medium alone (CON). Haem oxygenase activity was measured as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. CON.

7.4.2 Effect of hydroxyurea on haem oxygenase activity in K562 cells

Since hydroxyurea induces haem oxygenase activity in endothelial cells and modulates erythroid cell function, its effect on haem oxygenase activity in K562 cells was tested. K562 cells incubated with hydroxyurea (100, 300 or 500 μM) for 24 h exhibited a slight increase in haem oxygenase activity (Figure 7-3 A). At 500 μM concentration of hydroxyurea did not raise haem oxygenase, possibly because of cytotoxic effects at high concentrations.

It has been reported that a few days of exposure to hydroxyurea are needed to stimulate HbF production in erythroid progenitors (Fibach et al., 1993) suggesting that hydroxyurea causes a time-dependent adaptation in erythroid cells. Therefore, we thought it would be interesting to examine the time course of haem oxygenase activation after treatment with hydroxyurea. Figure 7-3 B shows that the levels of haem oxygenase activity fluctuated over time, even in cells exposed to medium alone, but the changes caused by hydroxyurea were more pronounced. In fact, 100 and 300 μM hydroxyurea promoted an early increase in haem oxygenase activity at 18 h and 24 h followed by a substantial fall at 48 and 72 h. Cells exposed to 500 μM hydroxyurea did not exhibit a significant increase in haem oxygenase activity compared to control at 18 and 24 h, and at 72 h the activity was markedly decreased below control levels. The literature reports only a few studies involving haem oxygenase and K562 cells. However, a specific investigation showed that thymidine (Trakshel et al., 1987), an inducer of haemoglobin synthesis in K562 cells, up-regulated haem oxygenase activity. To confirm these findings, K562 cells were exposed to 50, 100 and 200 μM thymidine for

24 h and it was observed that the compound induced haem oxygenase activity in a concentration-dependent manner (Figure 7-4).

Overall, the results show that hydroxyurea and thymidine can be used to increase haem oxygenase activity in K562 cells, in a mechanism still unknown but that could be related to the shared property of the two compounds to stimulate haemoglobin production in erythroid cells. However, even if thymidine and hydroxyurea up-regulate haem oxygenase, it is noted that the response of K562 cells is very contained, which is in contrast to the high haem oxygenase induction observed in other cell types upon exposure to the appropriate stimuli (Otterbein et al., 2003a).

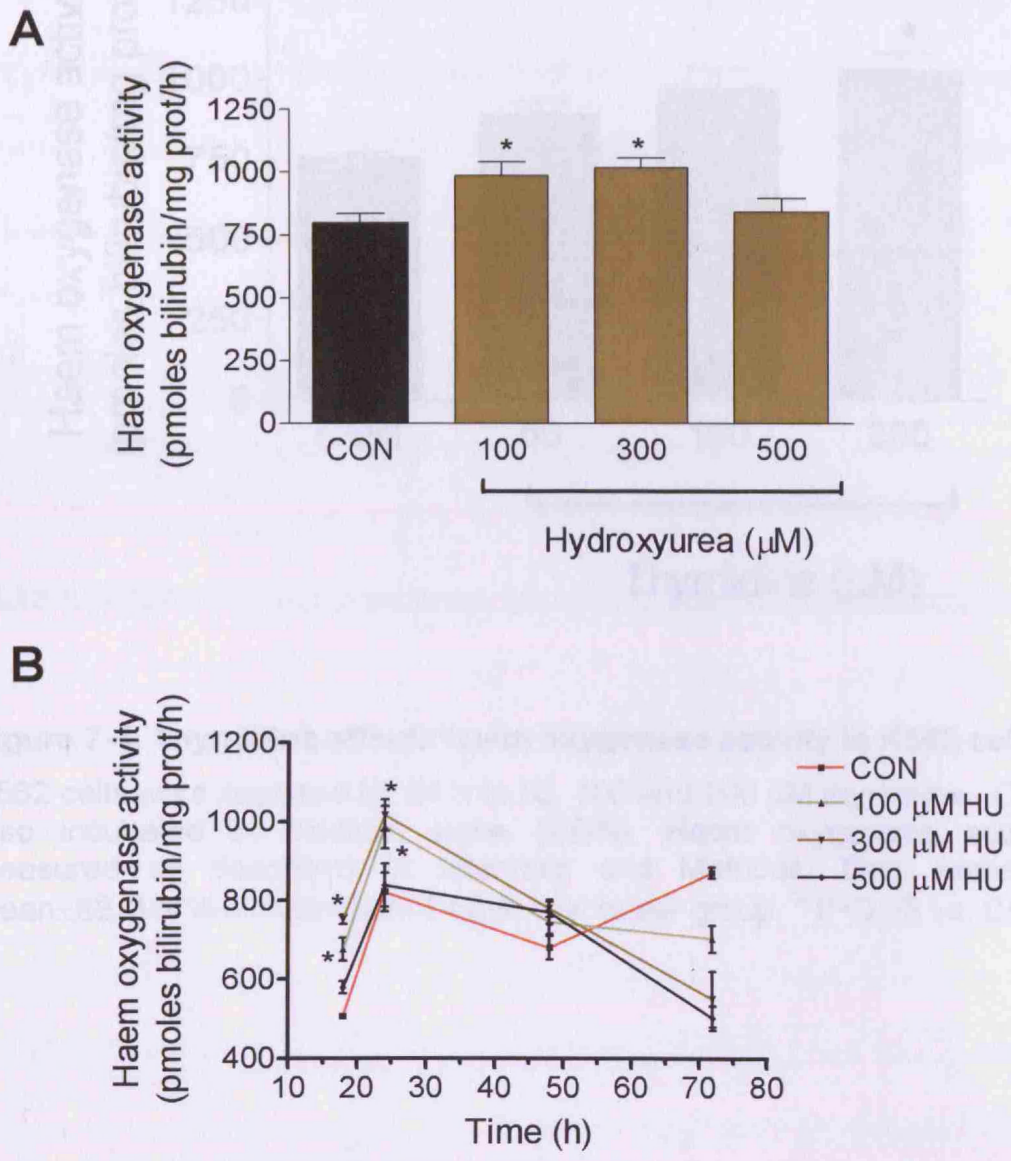


Figure 7-3. Hydroxyurea affects haem oxygenase activity in K562 cells: concentration and time-dependent effects

(A) K562 cells were exposed for 24 h to 100, 300 and 500 μM hydroxyurea (HU). (B) K562 cells were exposed for 18, 24, 48 and 72 h to 100, 300 and 500 μM HU. Cells were also incubated in medium alone (CON). Haem oxygenase activity was measured as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. CON.

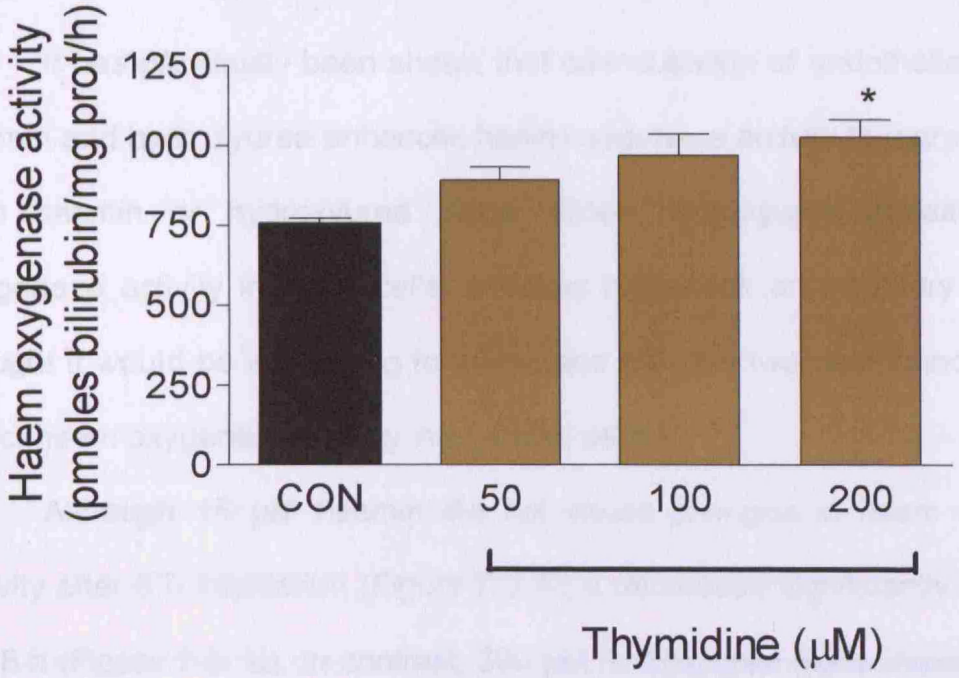


Figure 7-4. Thymidine affects haem oxygenase activity in K562 cells

K562 cells were exposed for 24 h to 50, 100 and 200 μM thymidine. Cells were also incubated in medium alone (CON). Haem oxygenase activity was measured as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. CON.

7.4.3 Co-incubation of K562 cells with haem and hydroxyurea: haem oxygenase activity response and cytotoxicity experiments

It has previously been shown that co-incubation of endothelial cells with haemin and hydroxyurea enhances haem oxygenase activity to a greater extent than haemin or hydroxyurea alone. Since hydroxyurea increases haem oxygenase activity in K562 cells, whereas haem has an inhibitory effect, we thought it would be interesting to investigate how the two compounds together affect haem oxygenase activity in erythroid cells.

Although 15 μM haemin did not cause changes in haem oxygenase activity after 6 h incubation (Figure 7-5 A), it decreased significantly the activity at 18 h (Figure 7-5 B). In contrast, 300 μM hydroxyurea alone increased haem oxygenase activity at both 6 h and 18 h. Interestingly, co-incubation of K562 cells with hydroxyurea and haemin showed a time-dependent inhibition of haem oxygenase induction.

These results indicate that when K562 cells are exposed to haemin and hydroxyurea, the haem oxygenase inhibitory action of haemin prevails over the activating effect of hydroxyurea. This is interesting as the opposite was observed in endothelial cells, and suggests that haem oxygenase may have a unique role in K562 cells.

Haem oxygenase has been shown to be protective against oxidative damage in various cell types. Considering the effects of haemin and hydroxyurea on haem oxygenase activity, we decided to investigate whether haemin and hydroxyurea affected the extent of cytotoxicity caused to K562 cells upon incubation with H_2O_2, a generator of oxidative stress. Figure 7-6 A shows that incubation of cells with 0.5, 1 or 2 mM H_2O_2 for 24 h resulted in

concentration-dependent cytotoxicity, with 2 mM H_2O_2 inducing damage equivalent to the positive control. One millimolar H_2O_2 produced approximately 60% cell damage and was the concentration chosen for the subsequent experiments with haemin and hydroxyurea.

Incubation of K562 cells with 15 μM haemin, 300 μM hydroxyurea or the combination of haemin and hydroxyurea for 18 h did not affect cell viability (Figure 7-6 B). Furthermore, pre-incubation with haemin, hydroxyurea or haem and hydroxyurea markedly reduced cytotoxicity caused by challenge with H_2O_2. These results suggest that haemin and hydroxyurea may be acting though haem oxygenase to protect K562 cells from oxidative damage.

To investigate this possibility, the haem oxygenase activity inhibitor SnPPIX was used. Figure 7-7 A shows that 15 μM SnPPIX significantly inhibited haem oxygenase activity of K562 cells incubated for 18 h with either medium alone or 15 μM haemin, 300 μM hydroxyurea or the combination of the two. Interestingly, the presence of SnPPIX did not influence the protection conferred by haem and hydroxyurea against H_2O_2–mediated cytotoxicity (Figure 7-7 B). Collectively, these findings suggest that the mechanism by which haemin and hydroxyurea protect K562 cells from oxidative damage may be haem oxygenase-independent, or that the residual haem oxygenase activity measured after incubation with SnPPIX may be sufficient to provide protection against oxidative stress.

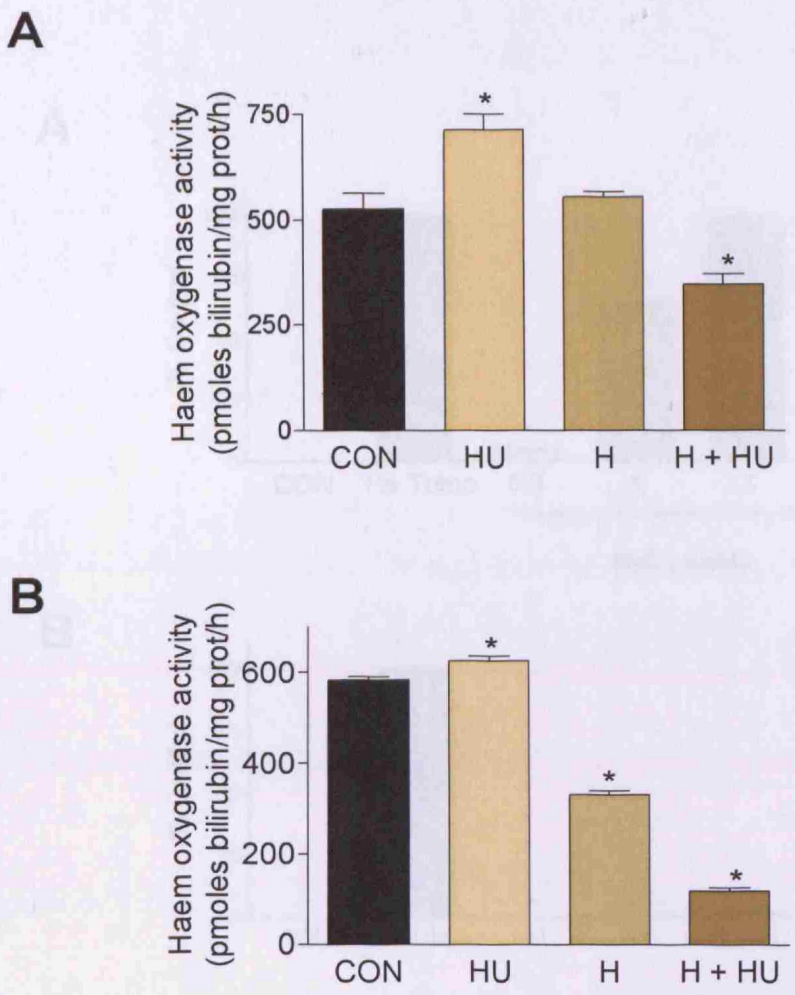


Figure 7-5. Hydroxyurea synergizes with haemin to down regulate haem oxygenase activity in K562 cells

(A) K562 cells were exposed to 15 μM haemin, 300 μM hydroxyurea (HU) or a combination of the two for 6 h. (B) K562 cells were exposed to 15 μM haem (H), 300 μM HU or a combination of the two (H + HU) for 18 h. Cells were also exposed to medium alone (CON). Haem oxygenase activity was measured as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. CON.

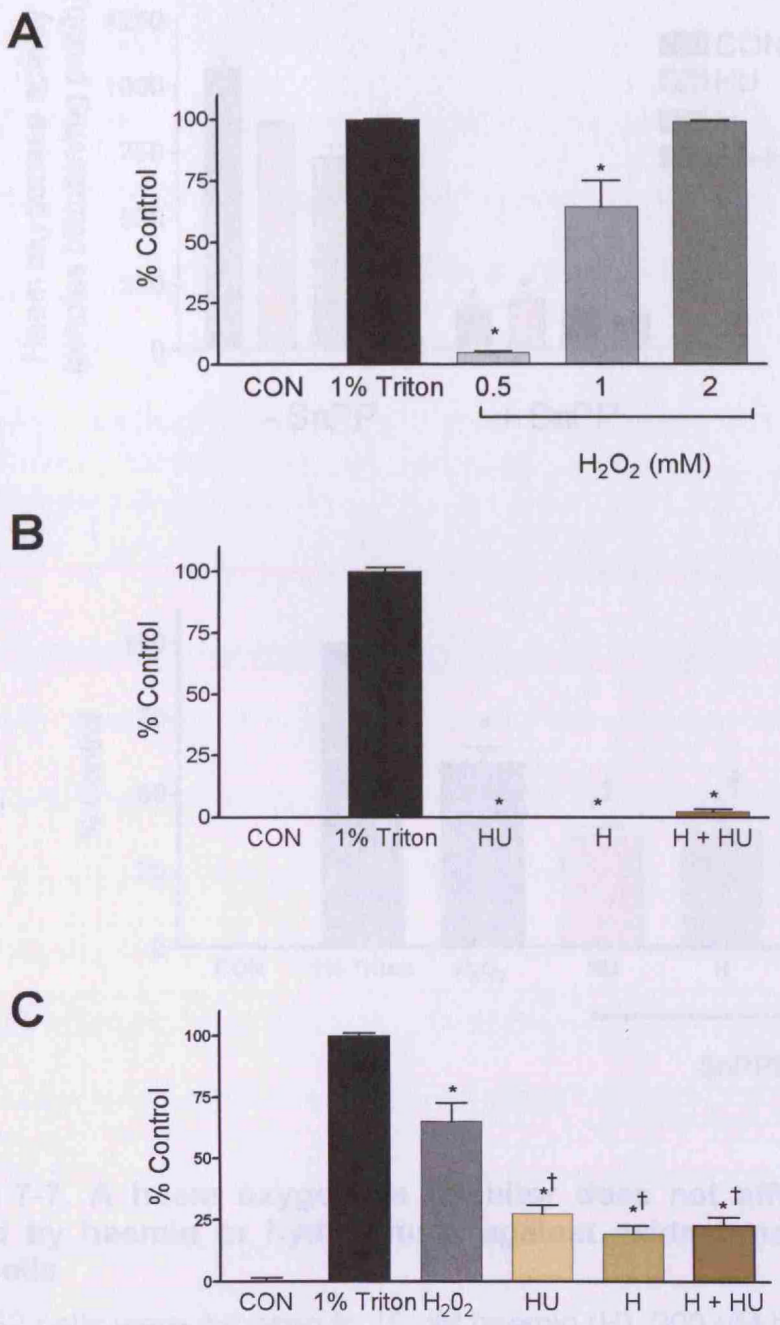


Figure 7-6. Haemin and hydroxyurea provide protection against oxidant-induced damage

K562 cells were exposed to: (A) 0.5, 1 and 2 mM hydrogen peroxide (H_2O_2) for 24 h; (B) 15 μM haemin (H), 300 μM hydroxyurea (HU) or a combination of the two (H + HU) for 18 h; (C) 15 μM H, 300 μM HU or a combination of the two for 18 h followed by 24 h incubation with 1 mM H_2O_2. Cells were also exposed to media alone as a negative control (CON) and 1% Triton as a positive control Lactate dehydrogenase levels were measured as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. 1% Triton. †$P<0.05$ vs. 1 mM H_2O_2 alone.

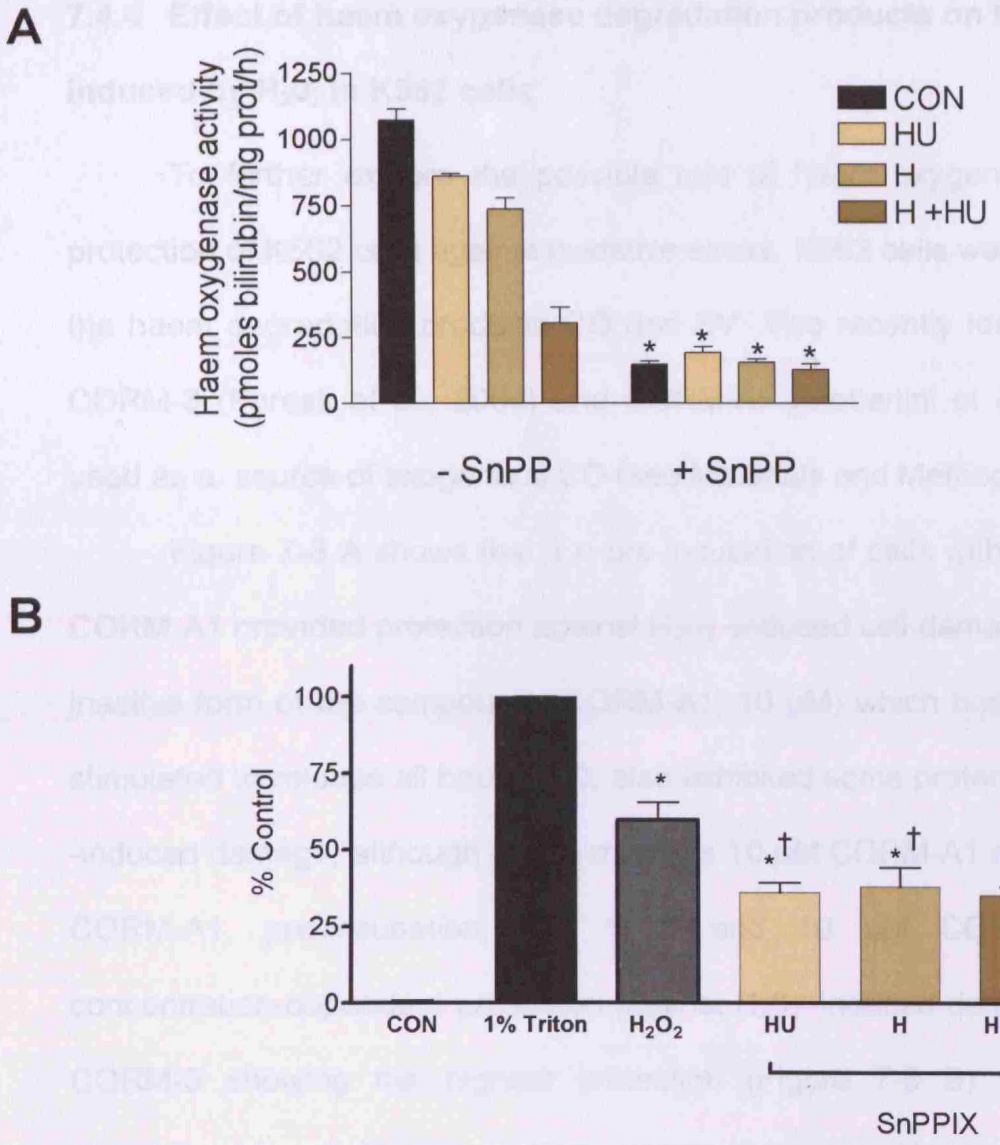


Figure 7-7. A haem oxygenase inhibitor does not affect the protection elicited by haemin or hydroxyurea against oxidant-mediated damage in K562 cells

(A) K562 cells were exposed to 15 μM haemin (H), 300 μM hydroxyurea (HU) or a combination of the two (H + HU) for 18 h, in the presence (+ SnPP) or absence of 15 μM SnPPIX (-SnPP), a haem oxygenase inhibitor. Cells were also exposed to medium alone (CON). Haem oxygenase activity was measured as described in Materials and Methods. (B) K562 cells were pre-incubated with 15 μM haemin, 300 μM HU or a combination of the two for 18h, in the presence of 15 μM SnPPIX prior to exposure for a further 24 h to 1 mM H_2O_2. Cells were also exposed to media alone (CON) as a negative control and 1% Triton as a positive control. Lactate dehydrogenase levels were measured as described in Materials and Methods Bars represent the mean±SEM of 4-6 independent experiments per group. * $P<0.05$ vs. -SnPP (A) or 1% Triton (B). †$P<0.05$ vs. 1 mM H_2O_2 alone.

7.4.4 Effect of haem oxygenase degradation products on the cytotoxicity induced by H_2O_2 in K562 cells

To further explore the possible role of haem oxygenase in providing protection of K562 cells against oxidative stress, K562 cells were incubated with the haem degradation products CO and BV. Two recently identified CO-RMs, CORM-3 (Foresti et al., 2004) and CORM-A1 (Motterlini et al., 2005c), were used as a source of exogenous CO (see Materials and Methods).

Figure 7-8 A shows that 1 h pre-incubation of cells with 1, 5, and 10 μM CORM-A1 provided protection against H_2O_2 -induced cell damage. However, the inactive form of the compound (iCORM-A1, 10 μM) which had been previously stimulated to release all bound CO, also exhibited some protection against H_2O_2 -induced damage, although not as much as 10 μM CORM-A1 alone. Similarly to CORM-A1, pre-incubation with 1, 5 and 10 μM CORM-3 elicited a concentration-dependent protection against H_2O_2 -induced damage, with 10 μM CORM-3 showing the highest protection (Figure 7-8 B). Again iCORM-3 showed some protection against oxidative damage, but not to the extent observed with active CORM-3. The effect of iCORM-A1 and iCORM-3 may be due to potential residual CO remaining in the molecule. Finally, our results indicate that also 1 h pre-incubation with 0.1, 0.5 and 1 μM BV significantly defended cells from cell damage induced by H_2O_2 challenge.

The above findings sustain the idea that low concentrations of haem degradation products are effective in providing protection against oxidative damage in K562 cells.

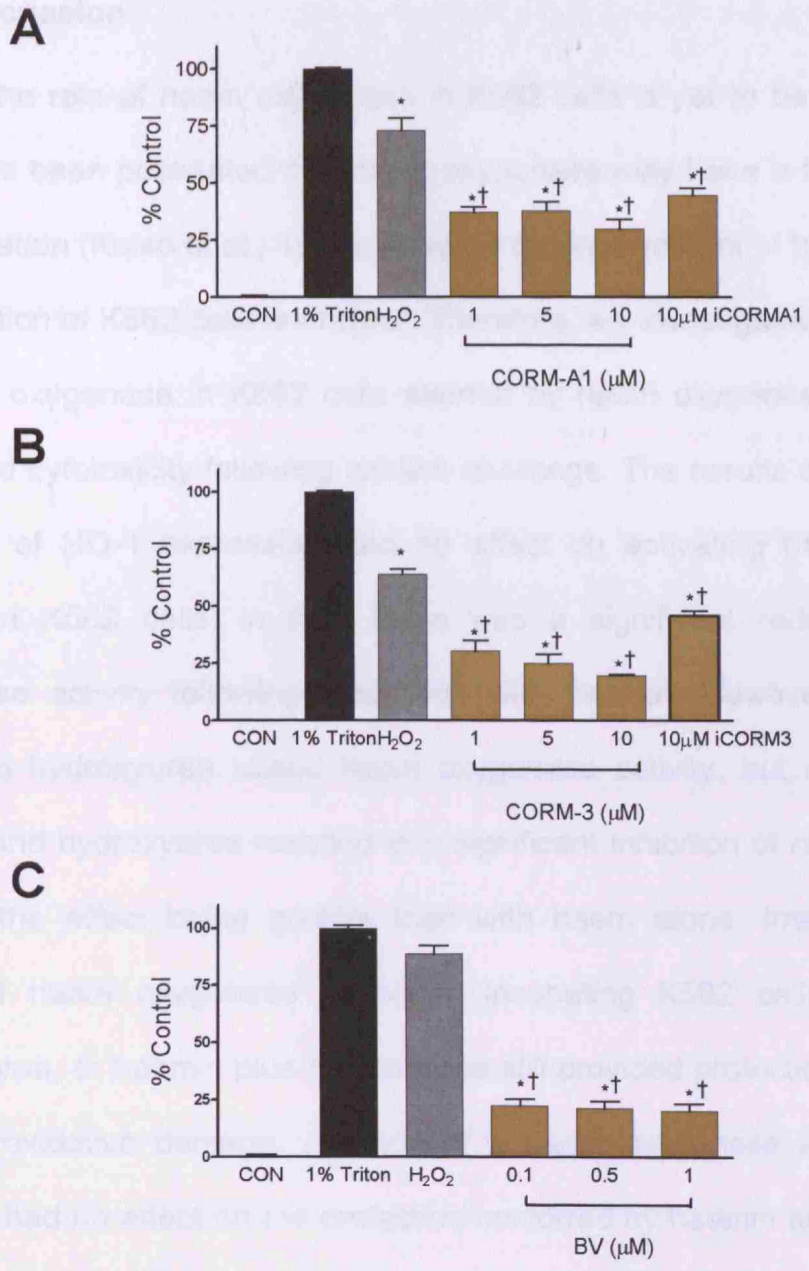


Figure 7-8. CO and BV protect K562 cells against oxidant-induced damage

K562 cells were pre-incubated for 1 h with: (A) 1, 5, 10 μM CORM-A1 or 10 μM inactive CORM-A1 (iCORM-A1); (B) 1, 5, 10 μM CORM-3 or 10 μM inactive CORM-3 (iCORM-3) and, (C) 0.1, 0.5, 1 μM biliverdin (BV), followed by exposure to 1 mM H_2O_2 for 24 h. Cells were also exposed to medium alone as a negative control (CON) and 1% Triton as a positive control. Lactate dehydrogenase levels were measured as described in Materials and Methods. Bars represent the mean±SEM of 4-6 independent experiments per group. *P<0.05 vs. 1% Triton. †P<0.05 vs. 1 mM H_2O_2 alone.

7.5 Discussion

The role of haem oxygenase in K562 cells is yet to be fully elucidated and it has been postulated that haem oxygenase may have a function in K562 differentiation (Koiso et al., 1999); however the involvement of haem oxygenase in protection of K562 cells is unclear. Therefore, we investigated the modulation of haem oxygenase in K562 cells exerted by haem oxygenase inducers and measured cytotoxicity following oxidant challenge. The results show that potent inducers of HO-1 expression had no effect on activating haem oxygenase activity in K562 cells; in fact, there was a significant reduction in haem oxygenase activity following incubation with haemin. However, treatment of cells with hydroxyurea raised haem oxygenase activity, but co-incubation of haemin and hydroxyurea resulted in a significant inhibition of haem oxygenase activity, the effect being greater than with haem alone. Irrespective of the observed haem oxygenase inhibition, incubating K562 cells with haemin, hydroxyurea, or haemin plus hydroxyurea still provided protection against H_2O_2-induced oxidative damage. Addition of a haem oxygenase activity inhibitor, SnPPIX, had no effect on the protection conferred by haemin and hydroxyurea, while pre-incubating cells with BV and CO mitigated oxidative damage. These data are difficult to reconcile and suggest that further investigation is required to determine if haem oxygenase is involved in the protection of K562 cells against oxidant-induced damage.

It is interesting that potent inducers of haem oxygenase expression such as haemin, curcumin and NO had no effect in inducing haem oxygenase in K562 cells. These results are in line with findings by Trakshel and co-workers (Trakshel et al., 1987), who previously reported inhibition of haem oxygenase

activity by haemin. BV and CO pre-incubation still provided significant protection from oxidant-damage, leading us to argue that despite the reduction in haem oxygenase activity by haemin, there is still enough residual haem oxygenase activity to confer protection to cells. It could be postulated that erythroid cells such as the ones used in our study would want to conserve haem for haemoglobin-synthesising purposes and thereby the observation that haemin significantly inhibits haem oxygenase activity in K562 cells would be consistent with this theory. Haem is not only incorporated as a structural component of haemoglobin but can also increase the expression of globin as well as the enzymes of the haem biosynthetic pathway in erythroid cells (Tahara et al., 2004). The treatment of erythroid cells with haemin has also been shown to increase both the number of transferrin receptors and the intracellular level of ferritin. Therefore, haem plays an important role in the coordinated expression of several genes during the differentiation of erythroid cells and it would be important to protect it from degradation by haem oxygenase in these cells. K562 cells express higher levels of HO-2 than HO-1 (Ding et al., 2006). HO-2 may be able to sequester haem bound to specific cysteine and proline residues which act as haem binding sites, thus maintaining intracellular haem concentrations or ameliorate haem-mediated oxidative stress. Challenge with stressful stimuli such as hypoxia inhibits HO-2 activity, resulting in increased intracellular haem concentrations and release of sequestered haem (Ding et al., 2006). These observations sustain the importance of preserving haem levels in K562 cells as this biomolecule is essential for the function of these haemoglobin-synthesizing cells. Interestingly, addition of anti-oxidants have been shown to block erythroid differentiation in K562 cells (Iwasaki et al., 2006), suggesting that blocking an

enzyme such as haem oxygenase, which protects against oxidant damage, is advantageous to the function of these cells.

Incubation with hydroxyurea increased haem oxygenase activity in K562 cells. This observation may be explained by the mechanism of action of hydroxyurea. Its therapeutic action in SCD is through stimulation of HbF production, which reduces the concentration of HbS and lowers the rate of sickling of red blood cells (Steinberg, 2002). Hydroxyurea inhibits DNA synthesis and prevents haemoglobin from maturing and hence stunt cell differentiation (Sauntharajah and DeSimone, 2004). Since haem oxygenase has been shown to act as a switch in the differentiation of K562 cells into either erythroid or megakaryocytic cells (Koiso et al., 1999), it could be proposed that the induction of haem oxygenase by hydroxyurea may be an additional mechanism through which the drug stimulates HbF production by inhibiting erythroid cell maturation. The increase in haem oxygenase activity by hydroxyurea reached a maximal level at 24 h and declined thereafter. In a clinical situation, sickle patients would be under hydroxyurea treatment for a prolonged period of time, therefore they would be continuously exposed to the drug and have a consistent level of hydroxyurea in their blood (Davies and Gilmore, 2003). This could possibly maintain a raised level of haem oxygenase over time, which may help in the production of HbF due to inhibition of cell differentiation. An attempt was made to measure HbF production in K562 cells in our laboratory, but it was unsuccessful. It would have been interesting to examine if there was any correlation with haem oxygenase activity level and HbF production.

Co-incubation of haemin and hydroxyurea in K562 cells resulted in a further inhibition of haem oxygenase activity. This may be a mechanism by K562 cells to further protect the breakdown of haem as discussed earlier. The interaction of hydroxyurea with haemin may form a complex which may be more resistant to degradation than haem alone, thus enhancing the amount of haem that can be utilized in haemoglobin production. Other studies have shown that haem is protective in K562 cells, for example, haemin was found to defend cells against lead-induced toxicity (Sarkar et al., 2005) and there was an associated increase in haem uptake by K562 cells. Haemin treatment was also found to protect K562 cells against pharmacologically-induced apoptosis (Mayerhofer et al., 2004) and the authors demonstrated that addition of CO and BV also resulted in partial rescue of cells from apoptosis but not to the extent seen by haemin. This suggests that haem oxygenase may have a role in shielding cells against apoptosis, but haem may act in other haem oxygenase-independent ways to provide protection as well. This idea is confirmed by our experiments using SnPPIX which did not reduce the protection elicited by haemin and hydroxyurea.

In summary, it was observed that well-known potent inducers of haem oxygenase expression did not increase haem oxygenase activity in K562 cells and that incubation with haemin and hydroxyurea conferred protection against oxidative damage (Figure 7-9). It may be advantageous for these cells to keep haem oxygenase activity low to preserve intracellular haem. However, residual low haem oxygenase activity may be sufficient to defend against oxidative damage in these cells.

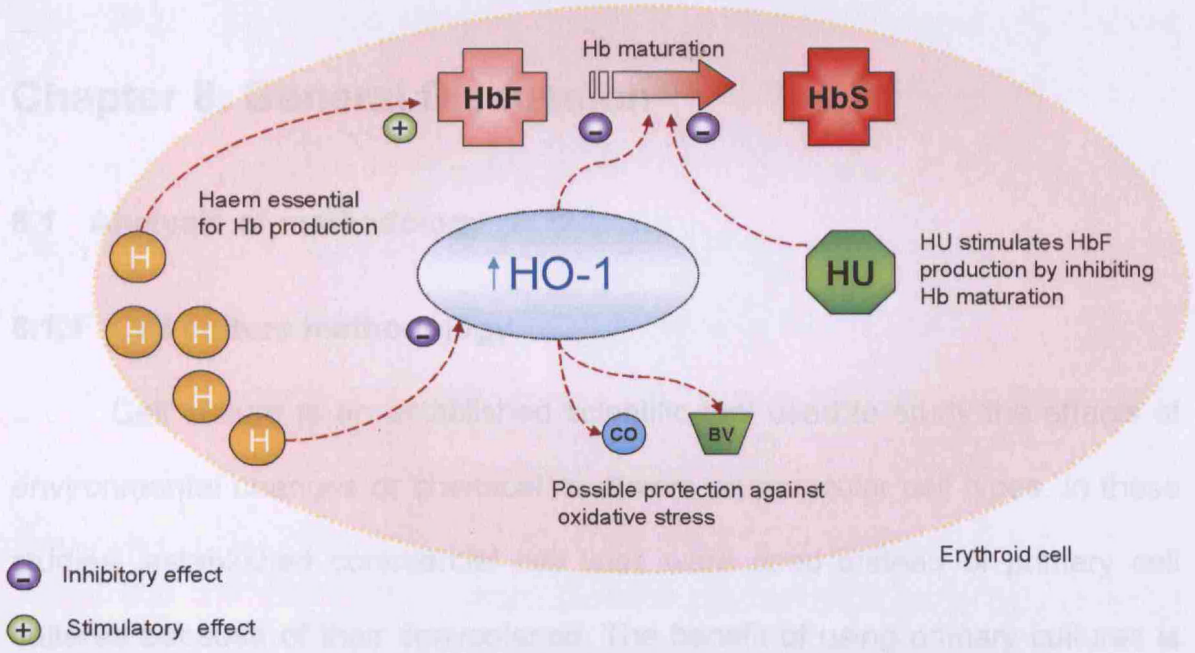


Figure 7-9. Potential role of haem oxygenase in K562 cells

The presence of haem (H) is essential to drive haemoglobin (Hb) production in erythroid cells and has an inhibitory effect on haem oxygenase-1 (HO-1). Hydroxyurea (HU) inhibits the maturation of HbS, resulting in the production of HbF. HO-1 may also inhibit haemoglobin maturation, as well as protecting erythroid cells from oxidative stress through the production of carbon monoxide (CO) and biliverdin (BV).

Chapter 8. General Discussion

8.1 Analysis of methodology

8.1.1 Cell culture methodology

Cell culture is an established scientific tool used to study the effects of environmental changes or chemical mediators on particular cell types. In these studies, established commercial cell lines were used instead of primary cell cultures because of their convenience. The benefit of using primary cultures is that they have not undergone modification (except by enzymatic or physical detachment); however, problems associated with primary culture include the presence of various cell types, limited culture lifespan and increased susceptibility to contamination. Therefore, commercial cell lines were utilised in the present studies as they are more stable and less labour intensive to maintain. The following cell lines were used: bovine aortic endothelial cells (BAEC), RAW 264.7 murine macrophages, human cardiac myoblasts (Girardi cells), renal epithelial cells (LLC-PK_1 cells) and human erythroid progenitor cells (K562 cells). In our studies, we explored the response of endothelial cells to various inducers of haem oxygenase, from haemin to whole blood, as well as haem uptake or other biological activities. BAEC were used as they have been established previously as an *in vitro* model to study endothelial haem oxygenase responses to oxidative stress (Foresti et al., 1999; Foresti et al., 2003; Ryter et al., 2000) as well as cellular haem uptake (Foresti et al., 2003). To explore if the observed effects of haemin, haemoglobin or NO on the endothelium could be replicated in other cell types we employed the use of

RAW 264.7 murine macrophages, Girardi cells and LLC-PK_1 cells. All of these cell types have been previously used to study the effects of oxidative stress on eliciting HO-1 responses (Abuarqoub et al., 2005; Abuarqoub et al., 2007; Balogun et al., 2003a). K562 cells are an established cell line widely used as an experimental model to study the molecular mechanisms of erythroid differentiation. The role of haem oxygenase in these cells has not been widely reported, although a few studies have shown modulation of haem oxygenase responses in K562 cells (Koiso et al., 1999; Koiso et al., 2000).

8.1.2 Biochemical and molecular biology techniques

The major biochemical and molecular biology techniques used were: (i) the haem oxygenase assay, (ii) Western blot analysis for HO-1 expression (iii), haem uptake assay (iv), BR measurement in culture medium (v), NO electrode and (vi) LDH assay.

8.1.2.1 Haem oxygenase assay

Tenhunen and co-workers (Tenhunen et al., 1969) first described the haem oxygenase assay in 1970 and this methodology has been used extensively in our laboratory (Foresti et al., 2003) to measure intracellular and tissue haem oxygenase activity. The assay determines the activity of haem oxygenase in the sample as picomole of BR per milligram of protein per hour. Because all substrates for the assay were added in excess, we measured the maximal haem oxygenase activity that could be stimulated in cells.

8.1.2.2 Western-blot analysis

Western blot is a recognized molecular biology technique for determining relative protein expression in cells and tissues (Laemmli, 1970); consequently,

every assay was run with both negative and positive controls (untreated sample and recombinant HO-1 protein). We use the Western blot analysis of HO-1 expression in combination with determination of haem oxygenase activity and the results we obtained were very consistent and reproducible. Each experiment is a representative blot of three independent experiments.

8.1.2.3 Determination of cellular haem

The determination of cellular haem content was measured using the protocol established by Balla and colleagues (Balla et al., 1991). This method has been widely used as a model to explore the incorporation of haem into various cell types (Regan et al., 2001; Uc et al., 2004) including endothelial cells (Balla et al., 2000; Foresti et al., 1999; Juckett et al., 1998). The assay gives a measurement of total cellular haem content and therefore does not provide information on location and utilisation of the haem incorporated. However, the assay is a simple and efficient tool to assess cellular haem content and used with appropriate controls delivers consistent and informative measurements on cellular haem incorporation.

8.1.2.4 Determination of bilirubin in culture medium

The determination of BR in culture medium was a method developed by Turcanu and colleagues (Turcanu et al., 1998) to assess the effects of oxidative stress on cellular haem oxygenase induction in *in vitro* work. Haem oxygenase activity can be quantified spectrophotometrically by measuring BR production from tissue or cellular microsomal fractions (Gemsa et al., 1974; Yoshida and Kikuchi, 1978); however the large number of cells required may be inconvenient for *in vitro* work. The detection of BR in the supernatant relies on the

quantitation of the BR released from cells into the culture medium and extraction of the bile pigment with benzene. Its advantages include its simplicity and the small number of cells required for every assay, since the extraction with benzene allows for a certain sensitivity.

8.1.2.5 NO electrode

The NO electrode (WPI, Sarasota USA) was used to measure the NO release from various NO donors in the presence or absence of haemoglobins. It is an amperometric sensor, where NO diffuses through a selective membrane covering the sensor and is oxidised at the working electrode, resulting in an electrical current. This electrode is a popular model and has been widely used for both direct measurement of NO and indirect measurement of both nitrite (NO_2) and nitrate (NO_3). The NO electrode and its associated ISO-NO meter (WPI, Sarasota USA) provides fast, accurate, and stable NO measurements over a wide range of concentrations in aqueous solutions. Advantages include rapid response to changes in concentration of NO, high sensitivity and selectivity and ease of use. It has been extensively used as research tool in exploring the release of NO from various NO donors (Gorren et al., 1996; Hurst et al., 1996) as well as exploring the effect of haemoglobin and NO interaction (Hakim et al., 1996; Schmidt et al., 1994).

8.1.2.6 Lactate dehydrogenase assay

An LDH assay kit (Roche Diagnostics, UK) was used as an indicator of cell viability. LDH is a cytosolic enzyme present within all mammalian cells, damage to cellular membranes results in leakage of LDH into extracellular fluid and is indicative of cell membrane damage (Drent et al., 1996). This LDH assay

kit is reported to provide a reliable method of assessing cytotoxicity in various cell types (Reeve et al., 2007; Song and Jeong, 2004; Tjoa et al., 2006) through *in vitro* release of LDH from cultured cells.

8.1.3 Endothelial cell adhesion assay

A static *in vitro* cell adhesion assay was used in the present studies to explore the interactions of red blood cells with endothelium. Ultimately, an *in vivo* model would be the favoured research tool; however it may be difficult to eliminate the effects of specific molecular pathways due to the complexity of the *in vivo* environment. Therefore, an advantage of *in vitro* models over *in vivo* cell adhesion models is that the experimental conditions can be modified to assess the influence of biochemical and biophysical variables (Shiu and McIntire, 2003). Static co-incubation of cultured endothelial cells and washed red blood cells first demonstrated the abnormally high adhesiveness of sickle red blood cells to endothelial cells (Hebbel et al., 1980b). The standard procedure for static assays involves allowing red blood cells to settle on and adhere to endothelial cell monolayers at 37°C for a period of time (gravitation sedimentation method (Shiu and McIntire, 2003)). Static assays provide a simple and rapid method to examine red blood cell-endothelial interaction and have generated significant insights into the molecular mechanisms behind abnormal cellular adhesion (Hebbel, 1997) and red blood cell-induced endothelial dysfunction (Hebbel et al., 1980a). However, while static assays reproduce regions of blood stasis within vessels, the data obtained from these assays may not be valid for the prediction of outcomes of red blood cells-endothelial interaction under flow conditions. A variation of static adhesion assays have been designed which incorporate fluid shear force to replicate flow

conditions (Smith and La Celle, 1986), but these assays are complex in practice.

8.1.4 Isolated aortic ring

Studies in isolated aortic rings are a useful tool to evaluate the pharmacological activity of a drug on the receptors, channels and enzymes of the vessel (Alm et al., 2002). Transverse sections of aortic rings are attached to tension transducers and suspended in buffer and changes in tension after administration of various compounds are measured. The growing use of this *ex vivo* model is due to the fact that it is inexpensive, requires fewer animals in comparison with *in vivo* models and allows the evaluation of the pharmacological activity of numerous compounds over a short period of time (Gebhardt, 2000). We employed the isolated aortic ring model to investigate the effect of vasodilators CO or NO in the presence of sickle blood. This model has been widely used to explore the effect of molecules such as CO (Motterlini et al., 2005c; Sammut et al., 1998; Sawle et al., 2006; Zhao et al., 2005) and NO (Irvine et al., 2007; Wanstall et al., 2001) on vessel relaxation. Furthermore, isolated aortic rings have been used to explore the interaction of haemoglobin (Crawford et al., 2003) and red blood cells on vessel relaxation (Mosseri et al., 1993). However, before extrapolating results obtained from the *in vitro* aortic ring model to the *in vivo* situation the following points should be considered. In the vasculature conduit arteries and resistance arteries differ importantly in size, function and local environment. Specifically, the aorta is a large conduit vessel and therefore has less influence on vascular tone than the smaller resistance arteries which play an essential role in the regulation of peripheral resistance. Thus, vascular reactivity data obtained from aortic tissue may not be an

accurate representation of physiological vessel tone. An alternative model that could be utilized to investigate contractility of the vasculature is small artery myography (Mulvany and Aalkjaer, 1990). This technique allows the study of smaller resistance vessels, for example, pre-capillary arteries, by mounting the vessel on two stainless steel wires passed through the vessel lumen. The subsequent isometric tension measurements are a closer approximation to physiological vascular reactivity than those observed with the aortic ring protocol. Small vessel myography also has the advantage of simultaneous measurement of internal vessel circumference, vessel wall tension, cell membrane potential, ion concentration as well as isometric responses.

8.1.5 Limitations of the study

Purified human haemoglobin was purchased from Sigma to use in experimental procedures. Ideally, we would have liked to purify haemoglobin from blood in our own laboratory, but due to lack of resources this was not possible. The purchasing of commercially available haemoglobin was a convenient and reliable alternative. We also relied on collaborators to produce haem-nitrosyl and CO-RM molecules for our experiments. Compounds were tested spectrophotometrically for purity and were kept under appropriate storage conditions to minimise degradation. However, we cannot guarantee some degradation did not occur after synthesis of the compounds.

We were fortunate to recruit sickle patients to donate blood for this thesis. However, the limited number of samples and small window of time in which we could use each sample restricted the number and scope of the experiments we could undertake. Furthermore, with only limited medical

background on each patient there is a possibility that other pathological processes, apart from SCD, may have contributed to our observed results.

The use of blood in our experiments also presented us with some technical limitations. Incubation of blood with endothelial cells for prolonged time periods led to sedimentation of red blood cells onto the endothelial layer. This did not cause problems for the measurement of haem oxygenase activity, as BR was directly extracted into chloroform, thus preventing any blood contamination. However, when attempting to measure HO-1 expression by Western blot analysis, the contamination of the sample with blood interfered with the blot. Similarly, haem uptake experiments with sickle blood were not possible due to the presence of red blood cells. We endeavoured to clean the samples but were unsuccessful in removing all the blood contamination. This is unfortunate as the effect of sickle blood on HO-1 expression and cellular haem uptake would have provided additional insights into the effect of sickle blood on the haem oxygenase pathway.

8.2 Hypothesis and objectives

The hypothesis for this thesis was:

Increased haem oxygenase activity and induction of HO-1 protein expression by interaction of haemoglobin and NO is an adaptive response of the endothelium and may have a physiological role in conditions such as sickle cell disease.

To verify this theory the objectives established in chapter 1 were:

- to investigate the effect of NO and haemoglobin interaction on endothelial haem oxygenase induction
- to examine the effect of NO and haemoglobin interaction on endothelial haem uptake
- to explore the effect of sickle blood on endothelial haem oxygenase induction
- to examine the effect of haem oxygenase and its breakdown products on sickle blood interaction with vascular endothelium
- to study the effect of HO-1 inducers on erythroid progenitor cells (K562)

The data presented in this thesis are consistent with this hypothesis and meet the objectives set. Chapter 3 established that interaction of haem/haemoglobin and NO increased the activity of HO-1 in endothelial cells and formation of an H-NO complex was presented as one of the possible

mechanisms underlying the enhanced HO-1 induction observed. The results reported in chapter 4 illustrated that interaction of haemin/haemoglobin and NO also increased the uptake of haem in endothelial cells with consequent accumulation of BR in the culture medium. Since this effect was seen in a variety of cell types, we suggested a novel role for NO in the modulation of haem transport. Sickle blood exposure was also observed to enhance endothelial haem oxygenase as a consequence of haemolysis in chapter 5. Interestingly, variables within the sickle patient population recruited for the study, such as time since last crisis and treatment regimen could modulate the extent of endothelial haem oxygenase activation elicited by sickle blood. Furthermore, hydroxyurea, the drug of choice in SCD and a NO donor, was demonstrated to activate endothelial haem oxygenase in the presence of haem or haemoglobin suggesting a potential new haem oxygenase-mediated mechanism of action for this drug. Haem oxygenase or its breakdown products were demonstrated to inhibit red blood cell-endothelial interaction in chapter 6 and CO still elicited vasodilation in the presence of sickle blood. These observations suggest a potential therapeutic action of haem oxygenase induction in SCD. The idea that hydroxyurea could modulate the activity of haem oxygenase was further strengthened in chapter 7, where a possible role for haem oxygenase in the action of hydroxyurea-induced HbF production in erythroid cells (K562 cell) was postulated as well as a possible contribution to cellular protection. The implications of all these findings will be explored further in the following section.

8.3 General discussion

Nitric oxide is a highly reactive molecule which can readily interact with various intracellular and extracellular components thus exerting many biological effects. In particular, much research and debate has gone into its interaction with haemoglobin and its consequent biological significance. The scavenging of NO by haemoglobin in haemolytic disease states such as SCD has been well established (Reiter et al., 2002) and results in vascular dysfunction. A physiological role for NO and haemoglobin binding has also been proposed, where interaction of haemoglobin with NO may help deliver the vasoactive gas to hypoxic tissue, either through formation of SNO-Hb or conversion of nitrite to NO by haemoglobin (Robinson and Lancaster, Jr., 2005). We have proposed a new role for haemoglobin and NO interaction where these two biological molecules act synergistically to induce the protective haem oxygenase system in endothelial cells. Our group has previously shown that NO increases haem-mediated induction of HO-1 by considerably enhancing the intracellular uptake of haem (Foresti et al., 2003). The up-regulation of HO-1 in the endothelial layer is essential as these cells are liable to be exposed to free haem following erythrocyte degradation. Therefore, enhanced uptake and degradation of haem by endothelial cells would limit haem-mediated pro-oxidant damage.

In the present thesis we also propose a novel role for the interaction of NO with distinct haemoglobins in the modulation of the haem oxygenase pathway, since co-incubation of HbA_0, methaemoglobin or HbS with NO donors results in increased endothelial cell haem uptake and HO-1 induction. These data suggest that NO may act to modulate the uptake of haem into endothelial cells, even though it is thought that haem transport in endothelial cells is not

regulated due to the inherent highly lipophylic nature of the tetrapyrrole ring. At present, no receptors for the transport of haem, haemopexin or haptoglobin have been found on the surface of endothelial cell membranes. However, if such receptors did exist, their activity could be modulated by NO. Indeed, our results have shown that pre-incubation of endothelial cells with a NO donor greatly enhanced the ability of cells to uptake haem. Therefore, NO may have an action on the surface of the endothelial cell membrane, either by affecting the binding properties of potential haem transport proteins (receptors or carrier proteins), or having a direct effect on the permeability of the cell membrane to enhance the direct incorporation of haem in to the cell. Our results also show that albumin reduced the uptake of haem into cells. However, the addition of NO still enhanced haem uptake in the presence of albumin, suggesting that NO could interact with haem bound to albumin to enhance haem incorporation. It would be interesting to look at the effect of more specific haem/haemoglobin carrier proteins, such as haemopexin and haptoglobin, on haem uptake in endothelial cells.

Due to the high affinity of haem or haemoglobin for NO, it is not surprising that nitrosyl-haemoglobin (HbNO) or haem-nitrosyl (H-NO) complexes may also form when NO and haemoglobins are co-incubated. These complexes have been detected under physiological and pathophysiological conditions, for example, in the liver of rats after endotoxic shock (Davies et al., 2005) and we observed that H-NO enhances both the induction of HO-1 and the uptake of haem in endothelial cells compared to haem alone, suggesting that H-NO could act as a substrate for HO-1. Our experiments with the NO electrode demonstrate that the release of NO from NO donors is modulated in the

presence of HbA_0 and HbS and we suggest that this affects the induction of HO-1 when haemoglobin and NO are present simultaneously.

However, we note that studies have shown the accumulation of H-NO complexes in endothelial cells after exposure to haemin and NO (Juckett et al., 1998) and the authors propose that NO may act to sequester haem from HO-1 degradation by forming a complex which is difficult to metabolise. In contrast, we have observed that H-NO not only is incorporated into cells faster than haem, it also enhances haem oxygenase induction as demonstrated through amplified haem oxygenase activity and BR production. The differences between the effects of H-NO on HO-1 induction in the two studies may be explained by variations in the experimental protocol. Our observations lead us to propose that the H-NO complex forms under conditions of excess NO and haem to limit the potential oxidative damage resulting from exposure of endothelial cells to free haem as well as the potential oxidative and nitrosative stress caused by NO. The breakdown products of HO-1 would provide additional protection against cellular damage (Motterlini, 2005) and such a mechanism may take place in pathophysiological conditions characterized by excessive NO and haem release.

Although we observed that H-NO enhanced HO-1 induction and haem uptake compared to haemin alone, this may not be the only mechanism by which interaction of haemoglobin and NO resulted in the up-regulation of the haem oxygenase pathway. In fact, co-incubation of NO and haemin still produced a greater haem incorporation and haem oxygenase induction than H-NO, suggesting that a combination of factors might contribute to the results observed. For example, NO could act on potential endothelial cell membrane

receptors or membrane permeability in general and facilitate uptake of haem or H-NO into the cell. Furthermore, the reaction of NO with haemoglobin to produce methaemoglobin would result in more haem for HO-1 to degrade. All these potential mechanisms are depicted in Figure 8-1. We have shown that the enhanced uptake of haem into the cells is accompanied by an increase in the activity and expression of HO-1. The HO-1-derived products BV/BR, CO and iron are also expected to increase and could help further to prevent oxidative stress-induced injury.

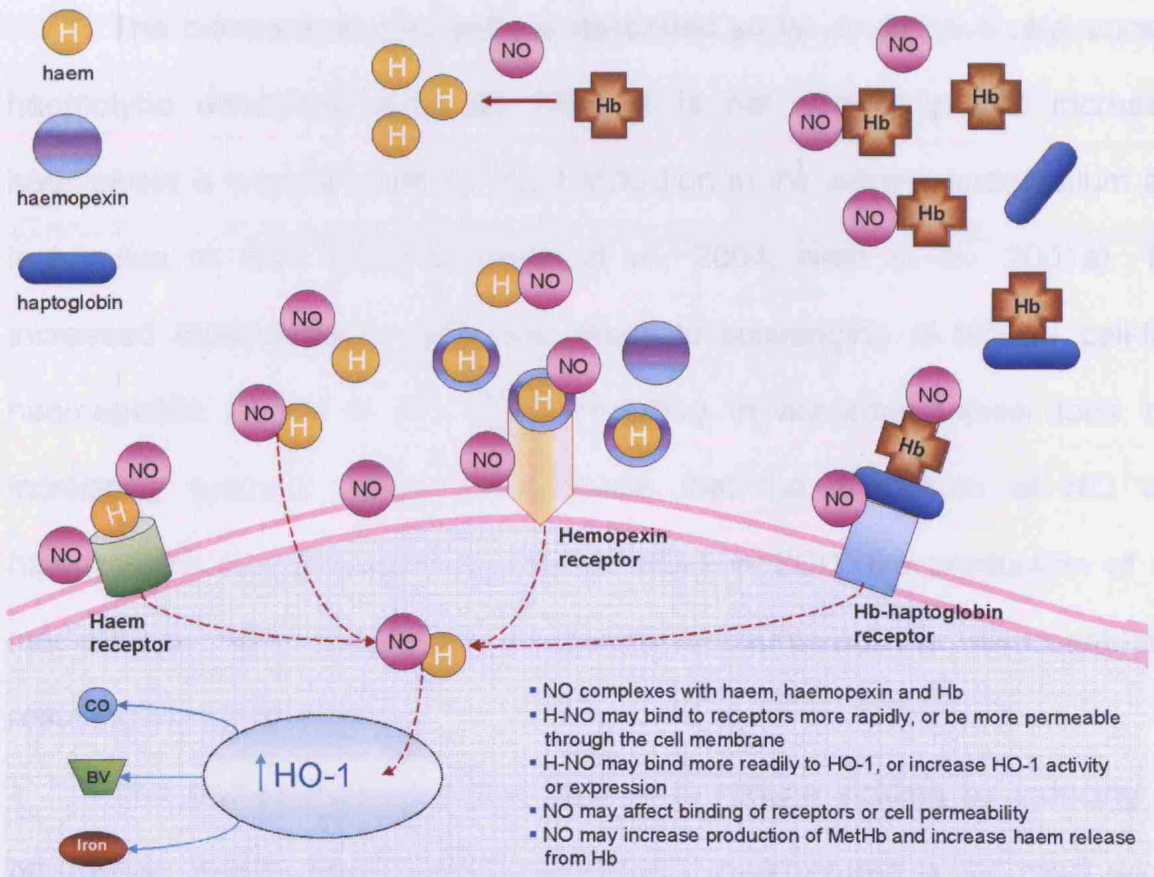


Figure 8-1. Possible mechanisms underlying enhanced haem oxygenase induction by NO, haemoglobin and haem

Nitric oxide (NO) can form complexes with haem (H), haemopexin or haemoglobin (Hb). The formation of haem-nitrosyl (H-NO) could allow easier transport into cells, where it would increase haem oxygenase-1 (HO-1) induction. Increased HO-1 activity results in the increased production of carbon monoxide (CO), biliverdin (BV) and iron.

Although endothelial cells, by virtue of being constantly exposed to haemoglobin/haem and NO, might have developed specific mechanisms to help them deal with the potential stresses caused by haem and NO, we have observed similar outcomes in other cell types. Specifically, renal epithelial cells, macrophages and Girardi cells showed to varying degrees an enhancement in the uptake of haem when co-incubated with haemin and NO. These results indicate that this synergism is not limited to endothelial cells and reinforces the idea of a possible regulation of haem transport by NO.

The biochemical mechanisms described so far could have relevance to haemolytic conditions such as SCD. It is not surprising that increased haemolysis is accompanied by HO-1 induction in the kidney, endothelium and leukocytes of SCD patients (Jison et al., 2004; Nath et al., 2001a). The increased haemolysis in SCD also leads to scavenging of NO by cell-free haemoglobin (Reiter et al., 2002), resulting in abnormal vessel tone and increasing vascular stasis. We propose that the interaction of NO with haemoglobin may promote induction of HO-1 in SCD; the production of CO mediated by haem oxygenase could help to counteract the vasoconstriction resulting from lack of NO.

The standard treatment for SCD is to reduce sickling by inducing the production of HbF (Bunn, 1997). Intriguingly, hydroxyurea is the most widely used compound to stimulate HbF production and has been shown to act as a NO donor (King, 2004). NO has also been used in other forms to alleviate the symptoms in SCD, for example through inhaled NO therapy (Gladwin and Schechter, 2001), or oral ingestion of the NO precursor arginine (Morris et al., 2003). We hypothesized that there may be a connection between HO-1

induction and hydroxyurea treatment in SCD. In fact, we observed that hydroxyurea enhanced the expression of endothelial HO-1 stimulated by haemin or haemoglobins. The NO-releasing properties of hydroxyurea could explain this effect; however, we did not observe an increase in haem uptake when endothelial cells were co-incubated with hydroxyurea and haemoglobin. Although hydroxyurea has been postulated to have NO donor properties, the mechanism of release of NO has yet to be fully elucidated (King, 2004). In *in vivo* settings high levels of HbNO, nitrates and nitrites are found in the blood of sickle cell patients receiving hydroxyurea treatment (Gladwin et al., 2002). We do not know whether we are able to replicate the conditions required for release of NO from hydroxyurea in our experimental protocol and our experiments measuring release of NO with the NO electrode do not support an important action of hydroxyurea as an agent that liberates NO. We speculate that NO could transfer from hydroxyurea to haemoglobin to form a complex which may explain the enhanced induction of HO-1 (Figure 8-2). However, more experiments are required to confirm this hypothesis.

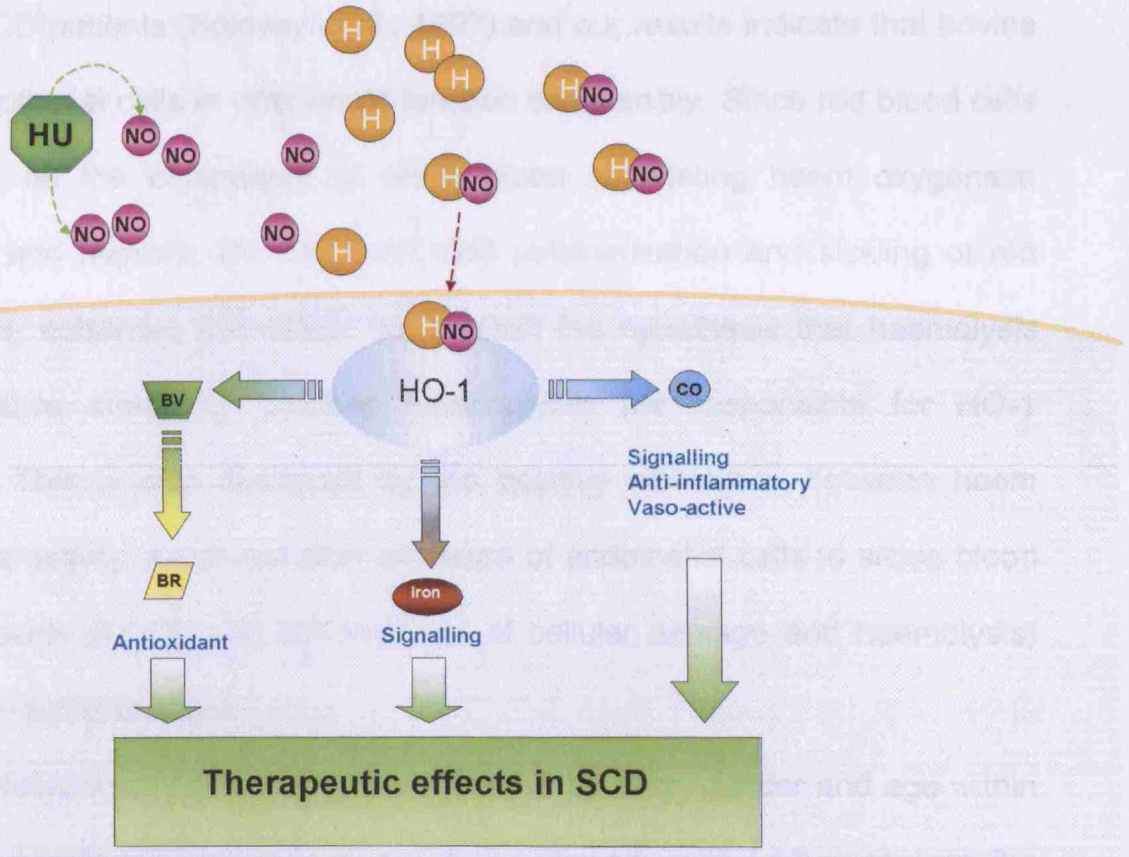


Figure 8-2. Potential effects of hydroxyurea on haem oxygenase in SCD

The release of nitric oxide (NO) from hydroxyurea (HU) could interact with cell-free haem (H), forming complexes which are more easily taken up by cells where they could stimulate haem oxygenase-1 (HO-1); this results in increased production of carbon monoxide (CO), biliverdin (BV) which is converted to bilirubin (BR), and iron. The biproducts of HO-1 could exert therapeutic effects in SCD through their vaso-active, anti-oxidant or anti-inflammatory properties.

Since raised haem oxygenase levels have been reported in various tissues in SCD (Jison et al., 2004; Nath et al., 2001a; Solovey et al., 1997), we wanted to investigate the effect of sickle blood on endothelial haem oxygenase. Sickle blood undergoes frequent haemolytic events which raise cell-free haemoglobin levels. In addition, high levels of pro-inflammatory molecules are present because of the chronic inflammation associated with the disease. These factors would be expected to affect HO-1 induction upon exposure of cells to sickle blood and this idea is confirmed by our findings in endothelial cells. Increased levels of HO-1 have been reported in circulating endothelial

cells of SCD patients (Solovey et al., 1997) and our results indicate that bovine aortic endothelial cells *in vitro* would function comparably. Since red blood cells appear to be the component of sickle blood stimulating haem oxygenase activation and hypoxia, the trigger of HbS polymerisation and sickling of red blood cells, enhances this effect, we support the hypothesis that haemolysis and oxidative stress by cell-free haemoglobin are responsible for HO-1 induction. This is also sustained by the positive correlation between haem oxygenase activity measured after exposure of endothelial cells to sickle blood and the levels of LDH and BR (markers of cellular damage and haemolysis) detected in patients blood.

Variations in treatment regimen, disease severity, gender and age within the sickle population recruited for our study allowed us to further analyse the relationship with endothelial haem oxygenase activation. Haem oxygenase activity levels were higher in cells incubated with blood from sickle patients who had a recent (in the last year) vaso-occlusive crisis, suggesting that the factors involved in haem oxygenase activation are present in blood for months after the occurrence of sickle crises.

Furthermore, experiencing 1-2 crises in the previous 2 years elicited stronger endothelial haem oxygenase responses than blood from patients who did not suffer crises or had greater than 2 crises. An increase in rate of crises in SCD is associated with increased mortality (Marchant and Walker, 2003) and lower haem oxygenase responses may be linked to a more severe disease state. There may be also a lack of factors responsible for the induction of haem oxygenase in blood from a subset of sickle patients. This could be linked to polymorphisms present in patients which may modulate the endothelial

response to oxidative stress. Indeed, genetic polymorphisms in SCD are possibly involved in the huge phenotypic heterogeneity witnessed in SCD (Elliott et al., 2007; Steinberg and Adewoye, 2006), suggesting that polymorphisms in genes for haem homeostasis such as HO-1, BVR, haptoglobin, haemopexin or CD-163 may modulate the cellular adaptation to haemolytic events. This is an important point that emphasises the fact that we did not measure haem oxygenase activity in blood of SCD patients. We instead assessed the ability of sickle blood to up-regulate the enzyme in endothelial cells in culture and these experiments give us an indication of the potential differences characterizing sickle blood and SCD pathologies that could influence haem oxygenase induction.

We report a significant positive correlation between haem oxygenase activity measured after exposure of cells to sickle blood from female patients and markers of haemolysis BR and LDH, a correlation which was not present in men. This data suggest that blood from SCD female subjects behaves differently when eliciting the haem oxygenase response compared to that of males, even though there was evidence of haemolysis in both genders. Interestingly, enhanced NO availability and NO responsiveness has been reported in female sickle patients (Gladwin et al., 2003) and as NO is a powerful inducer of haem oxygenase and synergises with haemoglobin to induce HO-1, the increased bioavailability of NO in blood from the female sickle population may explain the increased haem oxygenase responsiveness observed.

Blood from patients on hydroxyurea elicited a much lower endothelial haem oxygenase response than that of patients not receiving the drug. Since hydroxyurea treatment increases HbF production, the reduced haem oxygenase

response can be explained by a decrease in haemolysis due to stabilisation of red blood cells by the drug. However, we have also observed that haem oxygenase activity is raised in endothelial cells exposed to hydroxyurea, an effect which is magnified by the presence of haemoglobin and/or hypoxia. We propose that hydroxyurea may have both immediate and long-term therapeutic actions in SCD. In an acute crisis, the presence of free haemoglobin and hypoxia could act to enhance the effect of hydroxyurea on endothelial haem oxygenase induction with a consequent increased protection against oxidative stress. Long-term administration of hydroxyurea stimulates HbF production, diminishing sickling and haemolysis thus decreasing the oxidative burden exerted on the endothelium and resulting in lower haem oxygenase activity. The experiments with erythroid progenitor (K562) cells also indicate another therapeutic action of hydroxyurea potentially mediated by haem oxygenase. Hydroxyurea enhanced haem oxygenase activity in K562 cells and HO-1 seems to inhibit erythroid maturation in K562 cells (Koiso et al., 1999). We suggest that the induction of haem oxygenase stimulated by hydroxyurea in K562 cells may be implicated in the production of HbF mediated by hydroxyurea through inhibiting the synthesis of mature HbS. Further experiments with K562 cells showed that haemin and other classic inducers of haem oxygenase, as well as co-incubation of haemin with hydroxyurea inhibited haem oxygenase induction. Since haem is required for the synthesis of haemoglobin, erythroid cells may attempt to diminish its degradation by lowering the activity of haem oxygenase. Furthermore, erythroid cells could "sense" high exogenous free haem levels as an indicator of haemolysis and could develop adaptive feedback mechanisms to

conserve intracellular haem and stimulate haemoglobin synthesis in response to haemolytic events.

We also observed that haemin and hydroxyurea pre-incubation protected K562 cells against H_2O_2-induced cytotoxicity. However, a clear role for haem oxygenase was not established, since a haem oxygenase activity inhibitor did not reverse this effect but CO and BV exhibited cytoprotective actions. It is possible that the residual haem oxygenase activity measured following incubation with SnPPIX may be sufficient to elicit protection in K562 cells, but further investigation is required to elucidate this phenomenon.

In further experiments we wanted to determine whether haem oxygenase and its breakdown products could diminish pathological processes that are characteristic of SCD. We found that induction of haem oxygenase or its metabolites reduced sickle red blood cell adhesion to endothelial cells. Studies have reported that the adherence of sickle red blood cells may be due to increased expression of adhesion molecules (Brown et al., 2001) and oxidant production (Frenette, 2002) in sickle red blood cells. Furthermore, oxidative stress would also activate the endothelium to express adhesion molecules. Haem oxygenase could decrease adherence of sickle red blood cells because of the following: 1) HO-1 over-expression diminishes endothelial adhesion molecule expression (Soares et al., 2004); 2) BR, the reduction product of BV, has been shown to protect sickle cell membranes from oxidant damage (Dailly et al., 1998) and to reduce vascular endothelium activation and dysfunction (Kawamura et al., 2005); and 3) HO-1-derived CO exerts anti-inflammatory properties (Motterlini et al., 2003) that could diminish adhesion molecule expression by curtailing endothelial cell activation. It was interesting to observe

that pre-incubation of sickle blood with BV or CO-RMs reduced red blood cell adherence to a much greater extent than pre-incubation of endothelial cells. The effect elicited by CO-RMs might find an explanation in data reported previously by Beutler. This author showed that CO produced a right shift in the oxygen dissociation curve of HbS and decreased sickle haemoglobin viscosity (Beutler, 1974). In addition, Beutler also reported that treatment of sickle cell patients with exogenous CO gas prolonged red cell life-span (Beutler, 1975). These findings together indicate that sickle red blood cells could play a predominant role in promoting red cell adhesion suggesting they could be a candidate target for therapeutic intervention to minimise adherence in SCD.

Despite the increased levels of free haemoglobin which could scavenge CO, CO-RMs still elicited significant vasodilation in the presence of sickle blood (although relaxation was reduced in comparison to normal blood). This may be an important observation, as HO-1-derived CO may be able to counteract the vasoconstrictive effects caused by decreased NO bioavailability in SCD. In other words, the production of the vasodilator CO stimulated by haemoglobin (or the interaction of haemoglobin or NO) may substitute for the loss of NO, thus representing an adaptive mechanism evolved in haemolytic diseases such as SCD to counteract the deleterious effects of NO scavenging by haemoglobin on vascular tone. Interestingly, vessel relaxation studies also show that sickle blood enhanced the NO-induced vasodilation in comparison to normal blood. This is an intriguing observation, as the higher levels of free haemoglobin in sickle blood would suggest enhanced scavenging of NO.

Therefore, our findings suggest that administration of haem oxygenase inducers may offer a new strategy in the treatment of sickle patients via different

mechanisms (Figure 8-3). Alternatively, the therapeutic effects of haem oxygenase induction could be mimicked by exogenously administered CO or BV/BR, either individually or in combination. As both these molecules are toxic in high enough doses, further research is needed to determine safe and efficacious levels that could be used therapeutically. Intriguingly, the development of CO-RMs which bind and deliver CO to biological symptoms offers a potential new therapeutic approach to harness the beneficial actions of CO. Although further research is required, CO-RMs may provide an invaluable tool to liberate the powerful vaso-active, anti-oxidant and anti-inflammatory properties of CO in diseases such as SCD.

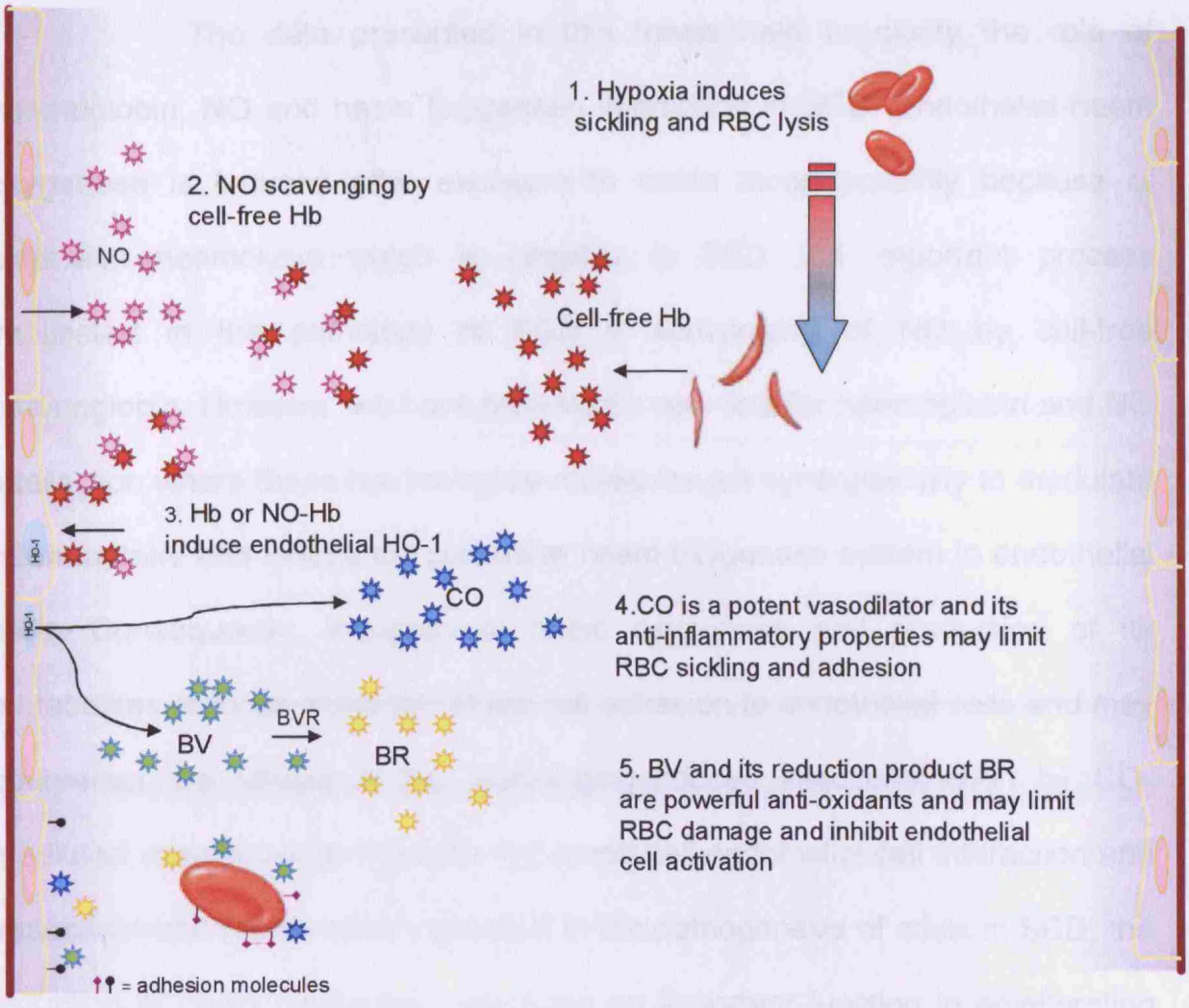


Figure 8-3. Haem oxygenase induction and potential therapeutic action in SCD

Hypoxia promotes red blood cell (RBC) sickling and lysis resulting in increased levels of cell-free haemoglobin (Hb) which can scavenge nitric oxide (NO). Hb or NO-Hb can induce endothelial haem oxygenase-1 (HO-1) resulting in the production of carbon monoxide (CO) and biliverdin (BV) which can be converted to bilirubin (BR) by biliverdin reductase (BVR). CO and BV/BR can modulate the haemolysis-mediated pro-oxidant and pro-inflammatory environment through potent, vaso-active, anti-oxidant and anti-inflammatory properties.

8.4 Conclusion

The data presented in this thesis help to clarify the role of haemoglobin, NO and haem oxygenase interaction in SCD. Endothelial haem oxygenase is induced after exposure to sickle blood possibly because of extensive haemolysis which is inherent in SCD. An important process implicated in the pathology of SCD is scavenging of NO by cell-free haemoglobin. However, we have proposed a new role for haemoglobin and NO interaction where these two biological molecules act synergistically to modulate haem uptake and induce the protective haem oxygenase system in endothelial cells. Consequently, induction of haem oxygenase and production of its metabolites reduces sickle red blood cell adhesion to endothelial cells and may counteract the effects of NO scavenging-induced vasoconstriction by CO-mediated vasorelaxation. As both red blood cell-endothelial cell interaction and vasoconstriction have been implicated in the pathogenesis of crisis in SCD, the induction of haem oxygenase may have an important function in ameliorating the pathology of SCD. Furthermore, haem oxygenase induction in endothelial cells and erythroid progenitor cells following exposure to hydroxyurea suggests a new haem oxygenase-mediated mechanism of action for this drug. Collectively, these findings indicate that modulation of haem oxygenase expression or administration of its breakdown products may offer potential new avenues in the treatment of SCD.

8.5 Future perspectives

HO-1 has been reported to act as a protective gene through the anti-inflammatory, anti-apoptotic and anti-proliferative actions of its breakdown products (Otterbein et al., 2003a). The observed interaction of NO and haemoglobin in regulating the expression of HO-1 as well as the haem oxygenase response to sickle blood offer intriguing insights for the understanding of the functional role of haem oxygenase in SCD and opens potential therapeutic avenues. However, further investigation is required to explore the potential implications of our findings.

- Further *in vitro* work is recommended utilising human endothelial cells instead of animal cell lines. Investigating the effect of haemoglobin and NO interaction in a human physiological model would strengthen the results obtained with BAEC.

- Due to the chronic inflammation inherent in SCD, investigating the effect of NO and haemoglobin interaction on haem oxygenase expression in macrophages would help to explore the role of haem oxygenase in modulating inflammation in SCD.

- The effect of NO or hydroxyurea on cell adhesion would provide invaluable additional insight into NO-haemoglobin interaction and its possible effects on red blood cell-endothelial adhesion. Furthermore, investigating the effect of hydroxyurea on cell adhesion may uncover another mechanism of therapeutic action for this drug.

- Additional vasorelaxation studies on aortic rings from animals pre-treated with haem, CO or BV would help to determine the effect of haem oxygenase-induction on vessel contractility in the presence of sickle blood. The treatment of animals or rings with H-NO complexes could also offer explanations for the observed effects of NO donors in the presence of sickle blood.

- Transgenic sickle mice are commercially available and would provide a useful model to study HO-1 expression in endothelium and other tissues. The effect of increased haem oxygenase activity on pathological processes such as cell adhesion and vasoconstriction could also be further investigated.

- Further investigation is required to explore a possible link between hydroxyurea-mediated haem oxygenase induction and HbF production in K562 cells. HbF measurements in K562 cells after the induction of haem oxygenase by hydroxyurea or other inducers of haem oxygenase in K562 cells may help clarify the situation.

Chapter 9. List of publications

1. Foresti R, **Bains S**, Sulc F, Farmer PJ, Green CJ and Motterlini R (2006) The interaction of nitric oxide with distinct hemoglobins differentially amplifies endothelial heme uptake and heme oxygenase-1 expression. J Pharmacol Exp Ther 317: 1125-1133.

2. Foresti R, Hoque M, **Bains S**, Green CJ and Motterlini R (2003) Haem and nitric oxide: synergism in the modulation of the endothelial haem oxygenase-1 pathway. Biochem J 372: 381-390.

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Part of the work presented in this thesis has been submitted recently for peer-reviewed publication as the following paper:

5. **Bains SK**, Foresti R, Howard J, Atwal S, Green C and Motterlini (2007) Human sickle cell blood modulates endothelial haem oxygenase activity in the endothelium: implications for vascular function and reactivity (submitted Blood Journal)

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Chapter 11. Appendix

11.1 Appendix 1: Full publications by the author